ANALYSIS OF THE GLUCOCORTICOID RECEPTOR IN SPONTANEOUS AND DRUG-INDUCED STEROID RESISTANT MUTANTS ISOLATED FROM THE HUMAN LEUKEMIC CELL LINE CEM-C7

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ABSTRACT

Title of Dissertation: Analysis of the Glucocorticoid Receptor in Spontaneous and Drug-Induced Steroid-Resistant Mutants Isolated from the Human Leukemic Cell Line CEM-C7.

Lisa A. Palmer — Doctor of Philosophy, 1991

Dissertation directed by: Jeffrey M. Harmon, Associate Professor, Department of Pharmacology

The mechanism of acquisition of glucocorticoid resistance in human leukemic T-cells was investigated. To assist in the analysis of the glucocorticoid receptor (GR), polyclonal anti-GR antibodies were prepared. An antibody, AP64, was made against a synthetic peptide sequence corresponding to Cys_{500}-Lys_{517} of the rat GR. AP64 reacted with the native and denatured forms of rat and human GR, preferentially interacted with the steroid-bound, activated, monomeric form of the GR, and blocked the binding of activated GR to DNA. Because the epitope for AP64 is known, the antibody defined a region of the GR that is occluded in the steroid-bound unactivated and non-steroid-bound forms.

The ability of adriamycin, bleomycin, and chlorambucil to induce mutations in the GR locus was examined using the steroid-sensitive human leukemic T cell line CEM-C7. Only bleomycin and chlorambucil were mutagenic, inducing steroid-resistance 2.5- and 5-6- fold above background, respectively. Biochemical characterization of the isolated drug-induced steroid-resistant cells demonstrated that the majority of the cells expressed the same $\underline{r}^-$ (receptorless) phenotype seen after mutagenesis with classical mutagenic agents.

Analysis of the steroid-binding activity and dissociation kinetics of [^{3}H]dexamethasone-labeled GR in cell extracts of sensitive and

resistant cells supported the proposed hypothesis that the GR in dexamethasone sensitive (dex^s) r^+ cells is encoded by two alleles, GR^+ and GR^*, while dexamethasone resistant (dex^r) r^- cells contain only the protein encoded by GR^*. The receptor encoded by GR^* rapidly loses steroid at 23°C, but could be stabilized by the addition of sodium molybdate. In addition, even in the presence of molybdate, this protein exhibited a faster rate of steroid dissociation than normal GR. These properties suggest that the GR^* allele contains a defect(s) in the steroid binding domain. Cells which were r^- showed decreased levels of GR protein and variable amount of GR mRNA. In addition, Southern blot analysis identified a deletion of a 4.5 kbp Bcl I fragment in 2 of 5 dex^r clones. This deletion identifies a defect introduced by mutagenesis of the GR^+ allele.

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by

Lisa A. Palmer

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Abbreviations and Definitions

act^1, the steroid-resistant activation labile mutant; Adr, adriamycin; Blm, bleomycin; CAT, chloramphenicol acetyltransferase; Chl, chlorambucil; d$^-$, the steroid-resistant deathless mutant; DEAE, diethylaminoethyl; dex, dexamethasone; DM, dexamethasone mesylate; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor, GR, glucocorticoid receptor; GR$^+$, the allele that encodes the functional GR protein in r$^+$ cells; GR*, the allele that encodes the non-functional GR protein in r$^+$ cells; GR$^-$, the mutated form of the GR$^+$ allele; HBSS, Hank's balanced saline solution; HEPES, 4-[2-hydroxymethyl]-1-piperazine-ethanesulfonic acid; HRE, hormone responsive element; hsp90, heat shock protein 90; K_d, equilibrium dissociation constant obtained from Scatchard analysis; KLH, keyhole limpet hemocyanin; k_{off}, the rate of steroid dissociation from receptor; MMTS, methyl methanethiosulfonate; MMTV-LTR, mouse mammary tumor virus long terminal repeat; MPL + TDM emulsion, monophosphoryl lipid A + trehose dimycolate emulsion; nt^i, the steroid-resistant nuclear transfer increased mutant; nt$^-$, the steroid-resistant nuclear transfer deficient mutant; PR, progesterone receptor; PRE, progesterone responsive element; Rs, Stokes radius; r$^-$, the steroid-resistant receptorless mutant; RNA, ribonucleic acid; RPMI 1640, Roswell Park Memorial Institute 1640; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TA, triamcinolone acetonide; TAPS, 3{[tris(hydroxymethyl)methyl]amino}-propanesulfonic acid; TCA, trichloroacetic acid

Antibodies

AC40:	polyclonal antihuman GR antibody made against the intact GR protein (Eisen et al., 1988)
AP64:	polyclonal antiGR antibody made against a synthetic peptide corresponding to Cys_{500}-Lys_{517} of the rat GR (Urda et al.,1989)
BuGR-2:	monoclonal antirat GR antibody (Harrison et al., 1984)

Buffers

Buffer B	25 mM TAPS, 1 mM EDTA, 10% glycerol, pH 7.6
Buffer 4	10 mM HEPES, 1 mM EDTA, 10% glycerol, pH 8.0
HEG buffer	10 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.6
Homogenization buffer	10 mM HEPES, 1 mM EDTA, 10 mM NaCl, 0.5 mM DTT, pH7.6
TAPS Buffer	25 mM TAPS, 1mM EDTA, 10% glycerol pH 9.5
2X Laemmli Sample Buffer	0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol

Cell Lines

Cell Line	Phenotype	Proposed Genotype
6TG1.1	dex^s, wt	GR^+, GR^*
ICR27TK.3	dex^r, r^-	GR^-, GR^*
3R7	dex^r, act^1	GR^{act1}, GR^*
IM-9	dex^s	GR^+, GR^+

INTRODUCTION

Steroid hormone receptors belong to a superfamily of transcription factors. This family includes receptors for the adrenal steroids (glucocorticoids and mineralocorticoids), the sex steroids (estrogens, progestins, and androgens), thyroid hormone, vitamin D_3, and retinoic acid. In addition, this family includes proteins whose ligands are yet unknown: estrogen receptor related proteins 1 and 2 (ERR1 and ERR2), hepatocellular carcinoma activating protein (HAP) and chicken ovalbumin upstream promotor (COUP) (Carson-Jurica et al., 1990; O'Malley, 1990). In general, each member of this family appears to have a similar mechanism of action and a similar structural and functional organization.

The generally accepted model of steroid hormone action involves diffusion of the steroid into the target cell, binding of the steroid to a soluble intracellular receptor protein, activation of the steroid-receptor complex, and finally, interaction of the activated steroid-receptor complex with specific DNA sequences (HRE's, hormone response elements) to affect the transcription rate of hormonally responsive genes. In some cases, the steroid receptor is primarily nuclear (estrogen, progesterone) (Carson-Jurica et al., 1990; Gustafsson et al., 1987). However, immunochemical localization of the glucocorticoid receptor (GR) has demonstrated it to be primarily cytoplasmic in the absence of hormone (Antakly and Eisen, 1984; Walters, 1985; Welchons et al., 1987, Wikstrom et al., 1987; Qi et al., 1990). Thus, for the GR, activation not only results in an increased DNA binding activity, but appears to trigger a change in the intracellular localization

of the protein. In any case, regardless of the site of localization, it is clear that in order for steroid hormone receptors to express their functional activity, ligand-specific binding, sequence-specific DNA binding, and transcriptional regulation are required.

I. Identification of Functional Domains

Biochemical analysis of steroid hormone receptors suggests that ligand-specific binding, sequence-specific DNA binding, and transcriptional activation are associated with specific structural domains. The structure of the GR was first identified by proteolytic digestion (Wrange and Gustafsson, 1978). The GR complex from rat liver cytosol was found to have an apparent Stokes radius of 6.1 nm. Treatment of the steroid receptor complex with α-chymotrypsin resulted in cleavage to a 3.6 nm form, whereas, treatment with trypsin resulted in 3.6 nm and 1.9 nm forms. In addition, the 3.6 nm chymotryptic fragment could be further digested with trypsin to form the 1.9 nm fragment. The intact 6.1 nm GR is immunoreactive with anti-GR antibodies (Carlstedt-Duke et al., 1982). This ability to react with anti-GR antibodies was only found in a 2.5 nm fragment, and was not found in either the steroid containing 3.6 nm or 1.9 nm proteolytic fragments (Carlstedt-Duke et al., 1982). The DNA binding activity found in the 6.1 nm GR was also found in the 3.6 nm proteolytic fragment, but not in the 1.9 nm proteolytic fragment (Wrange and Gustafsson, 1978). Steroid binding was present in the intact 6.1 nm protein, the 3.6 nm and the 1.9 nm proteolytic fragments. In experiments performed on purified, activated rat liver GR, where fragments were analyzed by SDS-PAGE,

similar results were obtained (Wrange et al., 1984). In this case, the purified protein migrates as three bands with the main band located at 94 kDa and two minor bands located at 79 kDa and 72 kDa. The 79 kDa band represents a putative degradation product (Wrange et al., 1984), while the identity of the 72 kDa species is unknown. Both the 94 kDa and the 79 kDa bands were found to bind steroid hormone and react with anti-GR antibodies. Proteolysis of the 94 kDa and the 79 kDa bands with α-chymotrypsin or trypsin resulted in the formation of a 39 kDa fragment which binds hormone and DNA-cellulose, but is no longer immunoreactive. The 39 kDa fragment formed could be further proteolyzed with trypsin into 27 kDa and 25 kDa fragments. However, additional treatment with α-chymotrypsin had no effect. Similar experiments were performed with [^{3}H]dexamethasone 21-mesylate (DM) affinity labeled GR from rat liver cytosol or from a transformed rat liver cell line (HTC) (Reichman et al., 1984). All of the proteases used generated fragments with relative molecular weights of approximately 42.5 kDa constituting the major DNA binding species, and two major steroid binding fragments (30.5 kDa and 27 kDa). No differences in the cleavage products of the unactivated and activated or the native or denatured receptors were detected. Thus, the functional activities associated with the GR: steroid binding, DNA binding, and immunoreactivity, appeared to be contained in distinct structural regions of the receptor protein, and activation of the steroid receptor complex did not appear to disrupt these proteolytic sites.

Molecular cloning of the GR from the rat (Meisfeld et al., 1986), mouse (Danielson et al., 1986), and human (Hollenberg et al., 1985) has confirmed this model and localized these activities. <u>In vitro</u>

expression and transcriptional analysis of normal and mutated GRs in connection with the various HRE's and reporter genes have been instrumental in obtaining information on steroid hormone-regulated transcription. In most of these experiments, a glucocorticoid responsive promoter/enhancer element, the mouse mammary tumor virus long terminal repeat (MMTV LTR), is linked to a "reporter" gene, such as chloramphenicol acetyltransferase (CAT), to form a "reporter" plasmid. An "expression" plasmid containing the sequence for the GR is co-transfected with the reporter plasmid into a receptor-negative cell. The expression plasmid provides the functional receptor which will allow the induction of CAT activity through the hormone-responsive promotor/enhancer element upon treatment with glucocorticoids. Insertions and deletions of specific regions of the GR sequence and the corresponding analysis of the function of these constructs in the presence of hormone were used to delineate the location of the functional domains. The structure of this protein was found to be divided into at least three functional domains: the amino terminal immunogenic domain (residues 1-393 human), the centrally located DNA-binding domain (residues 421-488 human) and the carboxyl terminal steroid-binding domain (residues 532-777 human) (Giguere et al., 1986). In addition, two regions were proposed to be involved in regulating transcriptional activation, τ_1 (residues 78-261 human), located in the amino terminal domain, and τ_2 (526-556 human), located at the amino terminal end of the steroid-binding domain. Thus, functional analysis of transfected deletion and insertion GR mutants has confirmed the earlier biochemical determination of functional domains, further delineated the location of these regions on the receptor gene, as well

as described additional regions thought to be important in normal GR function.

The amino terminal residues (1-393 human) are believed to be involved in modulating the receptor response. This is supported by the inability of a murine steroid-resistant mutant (nuclear transfer increase, $\underline{nt}^i$), which lacks the amino terminal 50 kDa as a result of aberrant RNA splicing between exons 1 and 3 (Dieken et al., 1990), to mediate cell killing in the presence of steroid. These $\underline{nt}^i$ mutants have an increased affinity for calf thymus DNA (Yamamoto et al., 1974; Yamamoto, 1976) and DNA containing a GRE (Pfahl et al., 1978a). In addition, analysis of the 39 kDa chymotryptic fragment of normal receptors shows that the receptor fragment contains a reduced ability to discriminate between specific and nonspecific DNA sites (Payvar and Wrange, 1984). This amino terminal domain has been found to contain a highly acidic region of amino acid sequence (residues 196-293 mouse) which when removed, increases the amount of nonspecific DNA binding activity (Danielson et al., 1987). Furthermore, the 39 kDa chymotryptic fragment binds to DNA as a dimer, like normal receptor, but with reduced specificity (Eriksson et al., 1990). Although footprinting assays detect no qualitative differences between the contact points of the 39 kDa chymotryptic fragment and the intact GR with specific DNA sequences, glutaraldehyde crosslinking experiments demonstrate differences in the protein-protein contacts of the two homodimers (Eriksson et al., 1990). Thus, loss of the amino terminal domain may affect steric arrangement and/or rigidity of the two DNA binding domains resulting in the decreased binding specificity.

It has also been suggested that the amino terminal domain contains the regions responsible for receptor antigenicity. The majority of the antibodies made against the intact GR protein are made to epitopes in this region (Rusconi and Yamamoto, 1987; Hollenberg et al., 1985; Westphal et al., 1984; Carlstedt-Duke et al., 1982; Smith and Harmon, 1987).

Analysis of the amino terminal domain has also found this region of the receptor to be necessary for full transcriptional activity (Danielson et al., 1987). Transcriptional activity was found in this domain by using a Lex A fusion protein containing 213 amino acids of the rat glucocorticoid receptor (residues 106-318), which enhances transcription, as well as a fusion protein containing the intact amino terminal domain of the GR (Godowski et al., 1988). A majority of the activity in this region, termed enh2, can be found in an 82 amino acid region consisting of residues 237 to 318.

The DNA binding domain (residues 421-488 human) is the centrally located cysteine-, lysine-, and arginine- rich region. This domain of the GR is the most highly conserved between species as well as between other steroid hormone receptors. Specific DNA sequences, known as HREs, are recognized by this domain. All HREs are palindromic and recognized by a dimeric DNA binding domain with two-fold symmetry (Berg, 1989). The glucocorticoid responsive element (GRE; AGAACAnnnTGTTCT) differs from the estrogen responsive element (ERE; AGGTACnnnTGACCT) by two nucleotides per "half site" (Klock et al., 1987). The thyroid-responsive "half site" (TGACCT) is identical to the ERE "half site", but the spacing between the "half site"s is different (Glass et al., 1988).

In this domain, there are nine conserved cysteine residues. Sulphur moieties of these cysteine residues are thought to coordinate a zinc atom to form zinc finger structures (Freedman et al. 1988; Hard et al., 1990a). The common sequence found in this region of steroid hormone receptors, cys-X_2-cys-X_{10-14}-cys-X_2-cys, is similar to the sequence found in Xenopus TFIIIA (Miller et al., 1985) and other transcription factors (Evans and Hollenberg, 1988). The amino terminal zinc finger of the rat GR contains cysteines 440, 443, 457, 460 (421, 424, 438, and 441 human) (Severne et al., 1988), while the second zinc finger is composed of cysteines 476, 482, 492, and 495 (457, 463, 473 and 476 human) (Severne et al., 1988; Hard et al., 1990a).

The DNA-binding domain largely determines the specificity of steroid hormone target gene action. Experiments in which the entire DNA binding region of the estrogen receptor was replaced with the corresponding DNA binding region of the GR ("finger swapping") produced a chimeric receptor with an estrogen-inducible glucocorticoid response (Green and Chambon, 1987). In similar "finger swapping" experiments, the amino terminal zinc finger, CI, was found to be responsible for determining the specificity of the receptor interaction, while the carboxyl terminal zinc finger, CII, stabilized the protein-DNA interaction by non-specific DNA binding (Green et al., 1988; Umesono and Evans, 1989; Danielsen et al., 1989; Mader et al., 1989). Substitution of the corresponding GR sequence into the CI region of the estrogen receptor and transcriptional analysis of the resulting receptor constructs have identified three amino acid residues of the estrogen receptor (glutamic acid, glycine, and alanine) which determine the specificity for an estrogen responsive element (ERE) verses a

glucocorticoid responsive element (GRE) (Mader et al., 1989). These three amino acid residues are located in the carboxyl terminal "knuckle" end of the first zinc finger. In a similar analysis of the GR containing amino acid substitutions from the DNA binding domain of the estrogen receptor, two amino acid residues (glycine and serine) located between the last two cysteines of the first zinc finger have also been found to allow discrimination of a GRE from an ERE (Danielsen et al., 1989).

Substitutions in the interfinger and second finger regions of the GR have led to chimeric receptors which can activate the transcription of both EREs and GREs. In vitro mutagenesis has determined that the $glycine_{439}$ to $glutamine_{439}$ substitution in the distal knuckle of the first zinc finger of the human GR will recognize both a GRE and an ERE (Umesono and Evans, 1989). In addition, replacement of five amino acids in the second zinc finger results in the recognition of a thyroid responsive element (Umesono and Evans, 1989). These experiments suggest that these amino acid residues present in the "knuckle" region of CI may be a primary determinant to target gene sequence recognition. However, the remainder of the DNA binding domain is important for conferring structural information required to prevent promiscuous HRE recognition.

Originally, the GR was thought to bind glucocorticoid-specific DNA elements in a 1:1 ratio (Wrange et al., 1986). However, recent evidence suggests that the GR interacts with DNA as a dimer (Tsai et al., 1988; Wrange et al., 1989; Hard et al., 1990b). Using gel retardation assays, a GRE/PRE of the tyrosine aminotransferase gene, and a peptide fragment consisting primarily of the DNA binding domain alone or bound to protein A, it was suggested that the binding of the GR to

the GRE/PRE occurred as a dimer (Tsai et al., 1988). Methylation interference suggested that the "half site"s were not equivalent, while gel retardation experiments using a heterodimer of the protein A bound peptide and the peptide fragment demonstrated that binding of the dimer occurred cooperatively, first to the downstream "half site" and then to the upstream "half site" (Tsai et al., 1988). Binding to the low affinity site appears to be dependent on the occupancy of the high affinity "half site", as well as the distance between the "half site"s and their orientation (Dahlman-Wright et al., 1990). Cooperativity of binding was also shown by fluorescence spectroscopy using an 115 amino acid protein fragment containing the DNA binding domain of the human GR and a 24 bp DNA binding oligomer containing the GRE for MMTV promotor region. Binding was shown to be consistent with a two site model (Hard et al., 1990b). The binding of purified preparations of activated (DNA binding) GR to a 35 bp fragment of the MMTV promotor(-189/-166), which contains a strong GR binding site, also confirmed this idea of dimerization. GR, DNA, and GR/DNA complexes were separated by glycerol gradients and showed that two hormone molecules were associated with each specific DNA site (Wrange et al., 1989). Formation of GR/DNA complexes was ligand dependent, yet, once formed, the GR/DNA complex was stable even after steroid dissociation (Wrange et al., 1989). Similarly, the estrogen receptor interacts with its cognate DNA sites as a dimer and binding to the DNA is ligand dependent (Kumar and Chambon, 1988). Although ligand binding is necessary for strong homodimer-DNA complex formation, the DNA binding domain has been suggested to contain a weak constitutively active dimerization signal since weak heterodimer-

DNA complexes can be formed between intact ER and truncated ER lacking the steroid binding domain (Kumar and Chambon, 1988).

Analysis of sequences present in the DNA binding domain have shown this region to contain transcriptional activation activity in addition to DNA binding activity (Hollenberg et al., 1987). Thus, this core DNA binding region contains a weak dimerization activity, is important in transcriptional activation, and in large part, determines the specificity of steroid receptor DNA interaction.

The carboxyl domain of the GR (532-777 human) contains the steroid binding activity. Several residues in this domain have been implicated in ligand binding. Cysteine 656 (rat) has been identified as the site of covalent interaction with the affinity ligand DM (Simons et al., 1987), while methionine 622 (rat) and cysteine 754 (rat) have been implicated in the binding of triamcinolone acetonide (Carlstedt-Duke et al., 1988). All of these residues are located in hydrophobic sections of the steroid binding domain. Proteolytic digestion of steroid-free rat GRs with low concentrations of trypsin indicates a single 16 kDa fragment can bind [^{3}H]DM (Simons et al., 1989). In addition, this fragment can bind dexamethasone with high affinity, although it is lower than that of intact receptor. This 16 kDa fragment, corresponding to amino acids threonine$_{537}$ to arginine$_{673}$, may represent a core ligand binding domain within the larger steroid binding domain.

Also contained within the steroid binding domain is a region of amino acids that are conserved among steroid hormone receptors (amino acids 577-596 human) and is called the transducing region (Pratt et al., 1988). This conserved sequence has been hypothesized to be the location for the interaction of the receptor protein with the receptor associated

protein, heat shock protein 90 (hsp90). The ability of anti-hsp90 antibodies to precipitate a 27 kDa steroid-binding tryptic fragment of rat liver GR supports the idea that hsp90 is associated with the steroid-binding domain (Denis et al., 1988). Further evidence confirming this interaction is that in vitro translated rat GR protein can bind hsp90 and this interaction occurs between amino acids 568 and 616 (Howard et al., 1990). It has been suggested that hsp90 binds to the "transducing" domain and may be necessary, but not sufficient, for keeping the steroid-binding site open and available for steroid occupancy (Bresnick et al., 1989). Upon ligand binding, a conformational change occurs increasing the rate of dissociation of hsp90 from the receptor.

Recent experiments using receptor chimeras consisting of the DNA-binding domain of the yeast transcription factor GAL4 joined to the steroid-binding domain of the human GR suggest that hormone binding is also at least partially responsible for receptor transformation and transcriptional activation (Webster et al., 1988). Elimination of the steroid-binding domain results in a constitutively active receptor, implying that the steroid-binding domain is responsible for the normal repression of transcriptional activity in the absence of a ligand (Godowski et al., 1987). There are two hypotheses for how the steroid binding domain can be involved in this repression. The "induction" model proposes that the steroid binding domain masks regions necessary for DNA binding and transcriptional activation. Upon binding of the ligand, a conformational change occurs, unmasking the sites required for DNA binding and transcriptional activation. The alternate hypothesis includes the interaction of the receptor with a receptor-associated

protein(s). Upon hormone binding, this protein(s) dissociates from the receptor and unmasks those sequences required for DNA binding and transcription. Transcriptional regulation by the steroid-binding domain, however, has been found to be independent of protein structure (Picard et al., 1988), thus supporting the second model.

To add to the complexity of the actions of the steroid binding domain, it is now thought that this region of the receptor is also important in the formation of stable receptor dimers since the formation of GR/DNA as well as ER/DNA complexes has been proposed to be ligand dependent under most circumstances (Wrange et al., 1989; Kumar and Chambon, 1988). Biochemical analysis of the subunit composition of the ER using partial tryspin proteolysis and dissociation of protein/protein interaction with the chaotropic salt NaSCN supports the idea that dimerization involves the steroid-binding domain (Sabbah et al., 1990). In these experiments, the 5S DNA binding form of the ER was found to be a homodimer containing two 66 kDa hormone binding subunits, whereas the steroid-binding tryptic fragment was found to be composed of two 30 kDa species that remain in the form of a dimer. This suggests that the steroid-binding domain was involved in dimer formation. In addition, a region required for receptor dimerization and high affinity DNA binding of the mouse ER has been defined (Fawell et al., 1990). This region (Arg_{507} -Ile_{518}) contains heptad repeats of hydrophobic residues which is conserved in all members of the nuclear receptor family. Using gel shift assays and site-directed mutagenesis, it was discovered that single amino acid substitutions of residues located in the amino terminal half but not the C-terminal half of the heptad repeat prevented receptor dimerization, whereas, steroid binding activity was abolished

by point mutations in the center of the conserved region. In addition, a 22 amino acid peptide sequence, containing Arg_{507}-Ile_{518} was able to restore DNA binding activity to receptor mutants lacking most of the hormone binding domain (Lees et al., 1990). Taken together, this suggests that the steroid binding and dimerization domains overlap.

II. Genomic Organization of the GR Gene

The genes encoding the members of this receptor superfamily are large. The GR gene is composed of 10 exons spanning approximately 150 kb (Encio et al., 1990). Similarly, the ER gene consists of 8 exons which span a region greater than 140 kb (Ponglikilmongkol et al., 1988). The promotor region in the GR gene is similar to the promotor region in the PR gene, containing no "TATA" or "CAAT" boxes (Zong et al., 1990; Huckaby et al., 1987), unlike the ER gene (Ponglikilmongkol et al., 1988). However, both the GR gene and the PR genes contain multiple GC rich regions (Zong et al., 1990; Huckaby et al., 1987). Large exons 1 or 2 are seen in the androgen receptor gene (Faber et al., 1989), progesterone receptor gene (Huckaby et al., 1987), estrogen receptor gene (Ponglikilmongkol et al., 1988) as well as the glucocorticoid receptor gene (Zong et al.,1990). Exon 2 for the GR gene was found to contain the initiator methionine codon, other potential start sites, and all of the amino terminal domain up to the DNA binding domain (Zong et al., 1990). Like the other steroid receptor genes, the zinc fingers in the GR gene are also encoded by two separate exons, exons 3 and 4 (Encio et al., 1990). The remaining exons encode the hormone binding domain and the 3' untranslated regions.

III. Glucocorticoid Receptor Activation

In the absence of steroid, GRs are found in the cytoplasm of the cell. Treatment with steroid followed by steroid-receptor activation causes a redistribution of the steroid-receptor complex from the cytoplasm to the nucleus (Middlebrook et al., 1975; Schmidt and Litwack, 1982; Antakly and Eisen, 1984; Walters, 1985; Welchons et al., 1987; Qi et al., 1990). This redistribution suggests that hormone binding and activation alter the receptor to a form that can interact with DNA. The process of activation has been shown to occur in vivo under physiological conditions and to be rate limiting for nuclear binding (Munck and Foley, 1979; Markovic and Litwack, 1980; Miyabe and Harrison, 1983). Activation in vitro can be produced by elevated temperature, increased ionic strength, elevated pH, or gel filtration (Milgrom et al., 1973; Goidl et al., 1977; Bailly et al., 1978). Addition of sodium molybdate can block this activation process reversibly (Dahmer et al., 1981). Examination of the thermal activation of purified rat hepatic GR provides evidence that activation is a two step process (Schmidt et al., 1985). The first step is temperature-sensitive, can be blocked by the addition of sodium molybdate, and does not require a cytoplasmic factor(s) present in crude cytosol preparations. Temperature-mediated transformation of the GR results in the exposure of positively charged amino acids (Milgrom et al., 1973; DiSorbo et al., 1980), increases the protein's affinity for nuclei, purified DNA, or DNA-cellulose (Milgrom et al., 1973; Baxter et al., 1972; Kalimi et al., 1975; Lefevre et al., 1979), and can be detected by altered DNA- and DEAE-cellulose profiles

(Parchman and Litwack, 1977). These changes could be due to a dissociation of a receptor-associated protein, such as the 90 kDa heat shock protein, whose association with the receptor cancels some of the GR's positive charge in addition to blocking the DNA binding site of the receptor. The second step of the activation process is temperature- and molybdate- insensitive, and requires the presence of a cytoplasmic factor(s) available in crude cytosolic preparations (Schmidt et al., 1985; Harmon et al., 1988; Cavanaugh and Simons, 1990a,b). This second step is believed to enhance the ability of the complex to bind to DNA-cellulose, but does not alter the DEAE-cellulose profile.

The effects of a recently described factor on the DNA binding activity of activated GR complexes supports the idea that GR activation is a two step process, at least in a subpopulation of activated GR complexes. This 700-3000 Da factor is found in cytoplasmic and nuclear extracts, is stable to heat, freezing, and thawing, does not bind to DNA, yet is required by some, but not all, activated GR complexes to bind to DNA (Cavanaugh and Simons, 1990a). These two populations of activated GR complexes can be distinguished by the effects of sodium arsenite and methyl methanethiosulfonate (MMTS); sodium arsenite blocks the ability of factor-dependent activated GR complexes to bind DNA while MMTS blocks the DNA binding activity of factor-independent activated complexes (Cavanaugh and Simons, 1990b). Although activation of both factor-dependent and factor-independent GR complexes can be blocked by molybdate, it has been proposed that activation of factor-dependent complexes occurs in two steps: the first step which can be blocked by molybdate, and the second step which requires the association of the DNA

binding activity factor and is insensitive to the effects of molybdate (Cavanaugh and Simons, 1990b).

Activation of the steroid receptor complex also results in a change in receptor size. Sucrose gradient centrifugation and size exclusion chromatography detect the steroid-bound unactivated GR as a 9S, 9-10 nm form and the steroid-bound activated receptor as a 4S, 4-5 nm form (Middlebrook and Aronow, 1977; Verdekis, 1983, 1983a; Sherman et al., 1983; Reker et al., 1985; Okret et al., 1985). Of these forms, only the 4S form binds to DNA. Analysis of the molecular interaction between the activated GR and DNA indicates that the activated GR interacts with DNA as a homodimer (Tsai et al., 1988; Wrange et al., 1989). Based on these determinations and the known size of the steroid binding protein, it has been calculated that the unactivated receptor is a multimer of 300-330 kDa while the activated receptor complex is a monomer and/or homodimer of 90-92 kDa. Analysis of unactivated (molybdate stabilized) murine L929 cell GR show the co-elution of two phosphoproteins from an affinity resin of deoxycortisterone agarose (Housley and Pratt, 1983). Both of these proteins can be co-extracted from molybdate-stabilized cytosol when incubated with anti-receptor monoclonal antibody and absorbed to protein A Sepharose (Housley et al., 1985). Characterization of these two proteins indicated that the 98-100 kDa murine protein could bind the affinity label DM, while the 90 kDa protein could not (Housley et al., 1985). Further investigation of the composition of the unactivated receptor multimer was performed using monoclonal antibodies directed against the 98 kDa steroid-binding protein. By comparing the relative molecular mass of the molybdate-stabilized GR in the presence of these anti-GR antibodies with the

relative molecular mass of the GR in the absence of anti-GR antibodies, only one anti-receptor antibody molecule was found to bind to one multimer. This suggested that the unactivated receptor protein contains only one 98 kDa steroid binding protein (Okret et al., 1985). The 90 kDa non-steroid binding protein was found to react with antiserum prepared against the 89 kDa chicken heat shock protein and was identified as the murine heat shock protein (hsp 90) (Sanchez et al., 1985). This 90 kDa non-steroid binding protein was found to also be associated with the molybdate-stabilized progesterone, androgen, and estrogen receptors (Joab et al., 1984; Sullivan et al., 1985), implicating this protein in steroid hormone action. Thus, one possible mechanism for GR activation is a dissociation of subunits from the unactivated, multimeric receptor complex to the activated monomeric DNA binding receptor complex (Vedeckis, 1983, 1983a; Sherman et al., 1983; Reker et al., 1985; Okret et al., 1985).

There are two hsp90 proteins associated with the steroid binding protein in the glucocorticoid receptor complex (Mendle and Orti, 1988). Without these proteins, steroid binding does not occur. Thus, the association of hsp90 with the GR may be required to maintain an appropriate steroid binding configuration (Bresnick et al., 1989). Hsp 90 is associated only with the molybdate stabilized, untransformed, 9S form of the GR (Sanchez et al., 1987a,b,c; Howard and Distelhorst, 1988). Under certain conditions, temperature-mediated dissociation of hsp90 is hormone-dependent. It also appears that hsp90 dissociates from the receptor complex in conjunction with the acquisition of DNA binding activity of the activated GR complex (Sanchez et al., 1987c). Moreover, ammonium sulfate precipitation of the receptor, which causes the

dissociation of hsp90 in the absence of steroid, converts the receptor to the DNA-binding form. Thus, hsp90 is one of the components of the heteromeric complex. It plays a role in maintaining the steroid binding configuration of the GR and helps to determine DNA binding activity.

IV. Factors Affecting GR Activation

Glucocorticoid receptor activation requires steroid-receptor interaction. Early studies suggested that sulfhydryl groups were important in the maintenance of steroid receptor binding activity. Oxidation of key sulfhydryl groups was believed to be responsible for the inactivation of steroid binding activity. The involvement of sulfhydryl groups in binding steroids in the GR in the rat thymus was shown by the use of various sulfhydryl protecting groups (Bresnick et al., 1988; Rees and Bell, 1975; Kobolinski et al., 1972.; Schaumburg, 1972). Sulfhydryl groups have been implicated in maintaining the GR in the proper configuration for binding ligand. The presence of thiols in the steroid-binding cavity of the GR has been shown by the affinity labeling of Cys_{656} in the steroid binding domain of the rat GR (Simons et al., 1987). Tritiated dex binding studies performed in the presence of methyl methanethiosulfonate (MMTS) indicate that there are at least 2 sulfhydryl groups involved in steroid binding (Miller and Simons, 1988). These sulfhydryl groups do not have to be in a reduced form for steroid binding to occur (Miller and Simons, 1988). Recently low concentrations of arsenite and cadmium (II) were also found to block steroid binding to the GR of HTC cells (Simons et al., 1990). The actions of these compounds on steroid binding are believed to be

mediated through reactions with vicinal dithiols.

Sulfhydryl groups are also involved in the transformation of the GR from the 9S non-DNA binding form to the 4S DNA binding form (Tienrungroj et al., 1987). Activation performed in the presence of MMTS inhibits the transformation to a DNA binding form. This inhibition can be reversed by the addition of dithiotreitol (DTT) (Tienrungroj et al., 1987). DTT cannot reverse the hydrogen peroxide inhibition of activation. Both MMTS and hydrogen peroxide prevent the characteristic shift of the 9S form to the 4S form and dissociation of the associated hsp90. Therefore, critical moieties are present in the untransformed receptor which must be in a reduced form for temperature-mediated dissociation to occur.

Sulfhydryl groups are also believed to be involved in receptor binding to DNA (Bodwell et al., 1984a). These sulfhydryl groups have been found to be different from those sulfhydryl groups required for steroid binding (Bodwell et al., 1984b); however, these groups may or may not be identical to those required for receptor transformation. The importance of sulfhydryl groups was discovered by the ability of sulfhydryl modifying reagents to inactivate the DNA binding of transformed receptors (Tienrungroj et al., 1987). MMTS will inhibit the DNA binding activity of the temperature-transformed rat GR. DNA binding activity can be restored after the addition of DTT. Hydrogen peroxide will also inactivate the DNA binding activity of the transformed GR and can also be reversed by DTT. Arsenite will also partially inhibit the DNA binding of activated complexes, although not to the extent of MMTS (Simons et al., 1990). Thus, it seems that sulfhydryl groups are important for the untransformed receptor complex to bind steroid, for

the steroid receptor complex to dissociate from hsp 90, for transformation, and for the transformed receptor to bind DNA.

Molybdate, a VI A transition-metal oxyanion, has also been shown to stabilize the unoccupied GR as well as inhibit GR transformation. Both actions are reversible and can be seen with other steroid hormone receptors. Molybdate stabilizes the GR in the 9-10S multimeric form and prevents the shift in the receptor to a less negatively charged state. The molecular mechanisms by which molybdate stabilizes the GR is unknown, but is thought to interact directly with the GR. Recent evidence shows that treatment of the GR with molybdate in combination with hydrogen peroxide will result in the irreversible loss of steroid binding activity. This loss is accompanied by a covalent modification of the receptor occurs as evidenced by a slight change in the apparent molecular weight on SDS-PAGE (Meshinchi et al., 1990). Pretreatment of the receptor with sulfhydryl modifying compounds, MMTS and N-ethylmaleimide, can prevent the covalent modification produced by the hydrogen peroxide/sodium molybdate combination. Further investigation using tryptic digests of the GR demonstrate that the location of the covalent modification occurs on a 27 kDa steroid binding fragment of the GR. Thus, modification of cysteine residues in the steroid binding domain may be involved in molybdate stabilization of the receptor (Meshinchi et al., 1990).

V. Cytolytic Effects of Glucocorticoids on Lymphoid Cells

Glucocorticoids interact with GR in almost all cells, producing a variety of effects. One such effect is the cytolysis of lymphoid

cells (Baxter et al., 1971). Although the mechanism of this action is unknown, one theory suggests that steroid-induced cell death occurs as a result of the induction of a "lysis gene" product (Compton and Cidlowski, 1987; Gasson and Bourgeois, 1983). The mouse cell line, SAK 8, responds to glucocorticoids by growth inhibition but not cell lysis. Treatment of these cells with 5-azacytidine, a DNA methylation inhibitor, results in glucocorticoid-sensitive clones that respond to glucocorticoids by cell lysis. This suggests that hypomethylation of a lysis gene(s) results in glucocorticoid-induced cell lysis. The induction of two protein families, a 30-32 kDa protein doublet and a series of 3-4 proteins of 12-19 kDa, upon glucocorticoid treatment, supports the idea of a lysis gene product(s). Both families were reported to have DNase activity which parallels the time course for glucocorticoid mediated DNA degradation. In addition, the induction of DNase activity could be blocked by the glucocorticoid antagonist RU 486 (Compton and Cidlowski, 1987). Unfortunately these protein products have recently been identified as H_1, H^o, and core histones (Alnemri and Litwack, 1989; Baxter et al., 1989). Therefore, the origin of the reported DNAse activity is unclear. Even though glucocorticoid induction of DNA fragmentation and cell death is not the result of _de novo_ induction of DNase activity, it is possible that activation of a constitutive endogenous endonuclease may be responsible (Alnemri and Litwack, 1989). Another theory suggests the repression of an essential gene product required for growth is responsible for glucocorticoid-induced cell death. Studies using murine S49, P1798 lymphoma cells, and human CEM C7 leukemia cells show glucocorticoids inhibit the transcription of cellular protooncogenes, particularly c-_myc_ (Eastman-

Reks and Vedeckis, 1986; Forsthoefel and Thompson, 1987). In addition, the synthesis of leukotriene B4 is inhibited by glucocorticoid treatment in murine S49.1 cells (Bittner and Wielckens, 1988). These glucocorticoid-induced growth inhibitory effects may be a prerequisite for cell cytolysis.

VI. Glucocorticoid Resistance in Lymphoid Cells

Given the numerous steps and the complexity of GR action, acquired steroid resistance could result from defects in hormone penetration, steroid binding, activation, translocation, and interaction with DNA. Various model systems have been developed to explore the mechanisms of acquired steroid resistance. Lymphosarcoma P1798 is a thymic lymphoma which becomes resistant to cytolysis in vivo as a function of tumor size (Lampkin and Potter, 1958). Steroid-resistant cells have been selected for resistance to glucocorticoid-induced cytolysis in vivo and in culture (Wood and Thompson, 1984). Steroid-resistant cells selected for resistance in vivo are indistinguishable from the wild type cells. The GR in these cells is fully functional and the cells are completely sensitive to the antiproliferative effects of glucocorticoids in culture (Wood and Thompson, 1984; Lucas et al., 1988). The basis for steroid resistance is therefore different from that seen in cells isolated for resistance to cytolysis in culture. The steroid-resistant cells isolated for resistance in culture harbor a receptor mutation. In these cells, there is little steroid binding activity (less than 500 binding sites per cell compared to 20,000 binding sites per cell in wt) defining these cells as having a

receptorless ($\underline{r}^-$) phenotype. In addition, there is no detectable immunoreactive receptor protein, DM-binding protein, or GR mRNA produced (Lucas et al., 1988). Data indicate that P1798 cells are functionally haploid and the cells isolated for resistance in culture have lost their ability to synthesize GR mRNA.

Glucocorticoid-induced cytolysis has also been utilized to select steroid-resistant variants from the cultured mouse lymphoma cell line, S49 (Sibley and Tomkins, 1974a). Of the steroid-resistant cells isolated, 80% contained defects in steroid binding, and are considered to be "receptorless" ($\underline{r}^-$). The other 20% were defective in the transfer of the steroid receptor to the nucleus and associated either with increased nuclear binding ($\underline{nt}^i$) or decreased nuclear binding ($\underline{nt}^-$). Some variants appeared normal except for the inability of steroid to produce cell death and these are referred to as "deathless" ($\underline{d}^-$) (Pfahl et al., 1977; Gehring and Tomkins, 1974; Sibley and Tomkins, 1974b). These spontaneously-derived steroid-resistant cells occur randomly in the steroid-sensitive population at a rate of 3.5×10^{-6}/cell/generation, consistent with mutation at a single locus (Sibley and Tomkins, 1974a). These steroid-resistant cells are stable in the absence of steroid and the steroid-resistant phenotype can be inherited from generation to generation (Sibley and Tomkins, 1974b). In addition, treatment of the steroid-sensitive population with mutagenic agents significantly increases the frequency of resistance. Somatic cell hybridization of wild type ($\underline{wt}$) and any class of steroid-resistant cell indicate each mutant phenotype is recessive (Gehring, 1980; Pfahl et al., 1978). Hybridization among the mutant receptor cells showed no complementation of receptor defects (Gehring, 1980). All of these

properties are consistent with somatic mutation as the cause for steroid resistance in these cells.

The GR gene in S49 cells is autosomal (Gehring, 1986) and functionally hemizygous (Bourgeois and Newby, 1977). Each cell produces a receptor which is non-functional in addition to the normal functional receptor protein. The non-functional protein is the result of a missense mutation at amino acid residue 546 resulting in a change from glutamine to a glycine (Danielson et al., 1986). The change results in a receptor protein which is immunoreactive but is unable to bind steroid since the change is located in the steroid binding domain of the gene.

The $\underline{r}^-$ mutant cells, by definition, are deficient in their capacity to bind steroid hormone. Immunochemical analysis of this phenotype revealed the presence of a 98 kDa immunoreactive protein which is indistinguishable in size from the wild type receptor. However, the quantity of this protein is only half the amount seen in $\underline{wt}$ cells (Northrop et al., 1985). Analysis of the respective GR mRNA from the $\underline{r}^-$ cells showed a decrease in the amount of normal 5 kb and 7 kb receptor mRNA produced (Northrop et al., 1986). The decreased message parallels the decreased level of immunoreactivity seen in these cells. Thus, the $\underline{r}^-$ cells fail to express a normal GR protein but continue to express the nonfunctional GR gene.

The $\underline{nt}^1$ receptor mutant has normal steroid binding activity. However, while immunochemical analysis of $\underline{nt}^1$ shows immunoreactive protein at 98 kDa, affinity labeling revealed a steroid binding protein with a M_r = 48 kDa. The 48 kDa receptor is unaffected by chymotrypsin treatment, which cleaves at amino acid residues 410-413 (rat) (Carlstedt-Duke et al., 1987), suggesting that an amino-terminal

truncation is responsible for the 48 kDa steroid binding species. This would account for its lack of immunoreactivity. In addition, receptor mRNA isolated from the $\underline{nt}^{i}$ cells revealed the presence of additional mRNA species at 5.5 kb and 3.5 kb, which appear to be a result of a 5' truncated message (Northrop et al., 1986). It has recently been shown that $\underline{nt}^{i}$ transcripts lack 404 amino-terminal residues as a result of aberrant RNA splicing between exons 1 and 3 (Dieken et al., 1990). As a result of the amino-terminal truncation, the $\underline{nt}^{i}$ mutant shows increased binding to non-specific (Yamamoto et al., 1974; Yamamoto, 1976) and specific DNA sequences (Pfahl, 1978a) which correlates well with the idea that the amino-terminal domain is important in modulating DNA binding activity. It also strengthens the idea that there are two alleles encoding the GR, in this case a 98 kDa non-steroid binding protein and a steroid binding 48 kDa $\underline{nt}^{i}$ mutant receptor protein.

Unlike the $\underline{r}^{-}$ and the $\underline{nt}^{i}$ receptor phenotypes, the $\underline{nt}^{-}$ phenotype has normal amounts of 98 kDa immunoreactivity and normal amounts of 98 kDa steroid binding protein. This mutant has been cloned and sequenced. A point mutation at amino acid residue 484, an arginine to a histidine change located in the DNA binding domain, has been found to destroy DNA binding activity (Danielson et al., 1986) Thus, it is clear that steroid resistance in the S49 cell line is the result of mutations in the receptor gene.

The human leukemic T cell line CEM-C7 is also sensitive to the cytolytic actions of glucocorticoids (Norman and Thompson, 1977). This cell line provides a model system to study the phenomenon of acquired steroid resistance in human cells. Three types of receptor phenotypes have been isolated from CEM-C7: $\underline{r}^{-}$ cells which contain almost no steroid

binding activity; act^1;molybdate sensitive cells, which are unstable during attempted activation but are stabilized in the presence of molybdate; and act^1;molybdate resistant, which are unstable during attempted activation and are insensitive to the affects of molybdate (Schmidt et al., 1980; Harmon et al., 1984a). Spontaneously arising mutants occur in CEM-C7 cells in the absence of selective pressure at a rate of 10^{-5}/cell/generation (Harmon and Thompson, 1981). These mutants all have reduced receptor levels and express one of the two act^1 receptor phenotypes. However, treatment of CEM-C7 cells with mutagenic agents results in GR mutants that are predominantly r$^-$. All of the steroid resistant-mutants, like the murine steroid-resistant cells, appear to be due to alterations of receptor function, are recessive, and do not complement each other (Harmon et al., 1985). These results indicate that each expressed phenotype is a result of a different mutation within the glucocorticoid receptor locus.

Like the S49 cells, the GR in CEM-C7 is autosomal (Gehring, 1986). Recent evidence suggests that the GR is coded by two GR alleles. One allele, GR$^+$, encodes for a functional GR protein that is able to bind steroid and is immunoreactive. The other allele, GR*, encodes a nonfunctional receptor protein which is unable to bind steroid under physiological conditions, but is immunoreactive (Harmon et al., 1989). In r$^-$ cells, the functional protein is no longer produced by GR$^+$, leaving the cells with only the nonfunctional protein. Analysis of the mRNA from the wild type and mutant cells showed normal 7 kb and 5 kb message being produced. However, in contrast to the case in r$^-$ mouse cells, no differences in the quantity of mRNA produced in the wt and the r$^-$ were noted (Eisen et al., 1988; Harmon et al., 1989). Thus, steroid

resistance in both S49 and CEM-C7 cells appears to be due to defects in GR function. However, the loss of GR function in r^- S49 cells is paralleled by the loss of GR mRNA which is not seen in r^- CEM cells. This suggests that the origins of steroid resistance in the two cells lines are different. Thus, in attempting to compare *in vivo* and *in vitro* steroid resistance, we chose to use the human T-cell line.

VII. Glucocorticoids and Leukemia

The property of glucocorticoid-induced cell death has led to the use of these steroids in the treatment of various leukemias and lymphomas (Claman, 1972; Goldin et al., 1971). Early clinical treatments using single agent glucocorticoid therapy were found to be effective in inducing initial remission. Unfortunately, remissions were short and relapses were frequent (Viette et al., 1965; Wolff et al., 1967). In addition, further glucocorticoid therapy was ineffective. To explain this resistance to steroid therapy, two hypotheses emerged. The first suggested that leukemic cell populations are heterogenous, containing both steroid-resistant and steroid-sensitive cells. With steroid therapy, the steroid-sensitive population is eliminated leaving the steroid-resistant population. The second hypothesis suggested that cells initially sensitive to steroid become resistant through mutation.

Modern therapies of leukemia and lymphoma utilize multidrug treatment regimens which commonly include glucocorticoids. Some of the other drugs used in the treatment of these lymphoproliferative diseases include adriamycin, bleomycin, and chlorambucil. All of these drugs interact with DNA. Adriamycin is an anthracycline isolated from strains

of Streptomyces peucetius and is known to intercalate with DNA (Myers, 1982). This intercalation is associated with detrimental effects on DNA synthesis, RNA synthesis and the creation of DNA damage. Single and double strand breaks occur and are believed to be due to the formation of free radicals during adriamycin metabolism. The semiquinone radical is believed to react with oxygen to yield superoxides and hydrogen peroxide which cleave DNA (Myers, 1982). Bleomycins are a family of glycopeptides isolated from culture broths of Streptomyces verticillis (Chabner, 1982). Bleomycin is believed to bind to guanine residues through the amino terminal tripeptide. The action of this peptide on DNA is to produce single and double strand breaks. Like adriamycin, these breaks are believed to be due to the production of free radicals. The Fe(II)-bleomycin complex may function as a ferris oxidase, catalyzing the reduction of molecular oxygen to superoxides or hydroxide radicals. The mechanism of chlorambucil action is different from those of adriamycin or bleomycin. Chlorambucil is an alkylating agent whose activity is elaborated through the covalent binding of alkyl groups to cellular molecules (Colvin, 1982). The predominant alkylation is the N-7 position of guanine. Interstrand and intrastrand DNA-DNA as well as DNA-protein crosslinks are believed to occur. Thus, these three drugs, used in combination therapy with glucocorticoids for the treatment of leukemia and lymphoma, are all potentially mutagenic. As a result, they may induce specific mutations in the GR locus that lead to steroid resistance.

If drugs used in combination protocols can induce mutations resulting in steroid-resistance, patients are potentially at risk to develop resistance to the steroid component of these protocols. Thus,

traditional combination therapies may not be ideal. Examining this problem in a model system employing cells susceptible to all four agents could, in principle, define whether this is a significant problem. The model system should be of human origin since differences in the origins of steroid resistance can be seen between mouse and human cells. In addition, the model system eliminates some of the complexity seen in multidrug therapy by studying the effects of one drug at a time on the development of steroid resistance. Furthermore, the model system should allow the isolation and characterization of steroid-resistant cells. The characterization of such cells can determine whether steroid-resistant cells isolated after different drug treatments have similar or different characteristics. If the characteristics are different, it might be possible to define a specific "signature" for each drug and thereby develop methods to monitor the acquisition of steroid-resistance in patients receiving ambitious therapies.

VIII. Objectives

From the above discussion, it is clear that the effects of steroid hormones are mediated by specific intracellular receptors through a complex series of steps. One specific effect mediated by the GR is the cytolysis of lymphoid cells of T cell origin. It is clear that mutations in the major domains of the GR can lead to steroid resistance. In addition, given the known capacity of cancer chemotherapeutic drugs to interact with DNA and induce mutations, it seemed reasonable to examine the ability of some of the drugs to induce mutations in the GR locus. Employing the steroid-sensitive human leukemic T cell line CEM-C7 as a model system, we examined the following: (1) the ability of the

drugs adriamycin, bleomycin, and chlorambucil to induce mutations rendering cells resistant to the cytolytic actions of glucocorticoids; (2) the biochemical basis of receptor defects in drug-induced dex^r cells; (3) the expression of the human GR protein and mRNA in dex^r cells; and, (4) the organization of the GR genes in dex^s and dex^r cells. To facilitate these studies and to gain additional information on the structure and organization of the GR protein, we attempted to prepare antibodies against the various domains of the GR.

Preparation of anti-peptide antisera

Female New Zealand white rabbits (Dutchland Laboratories, Denver, PA) were immunized at 4 week intervals over an 8 week period. Peptides conjugated to KLH by the manufacturer (Peninsula Laboratories, Belmont, CA) were suspended in 1 ml MPL + TDM emulsion (RIBI ImmunoChem Research, Inc., Hamilton, MT) and used to immunize rabbits at multiple (15-20) intradermal sites in the back. The total amounts of peptide-KLH conjugate used in the initial immunizations were 7.8 mg Thr_{173}-Gln_{189}, 2.9 mg Cys_{500}-Lys_{517}, 4.0 mg Asn_{637}-Met_{652}, and 8.0 mg Asp_{659}-Leu_{671}; subsequent immunizations used 7.9 - 8.3 mg of the conjugated peptides. Animals were bled 14 days after each immunization. Sera were tested for the presence of anti-peptide antibodies using dot immunoblot analysis (see below). Positive sera were further screened for specificity by immunoblot analysis of immunopurified IM-9 and HTC GR. Subsequently, animals were immunized twice with 8.0 - 11.6 mg of the conjugated peptides suspended in 1.0 ml Complete Freund's Adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). Rabbits immunized with Cys_{500}-Lys_{517} were immunized once more with 7.6 mg conjugated peptide suspended in Incomplete Freund's Adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). Positive sera were detected in two rabbits (AP63 and AP64) immunized with Cys_{500}-Lys_{517}. The highest antibody titer, determined by the specific absorption of steroid receptor complexes to Protein A, was observed in rabbit AP64 after the third immunization.

Dot Immunoblot

Peptide-KLH conjugates and KLH (Behring Diagnostics, La Jolla, CA) were solubilized and diluted to 1 mg/ml with phosphate buffered saline (PBS; 140 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.69 mM Na_2HPO_4 pH 7.2). Serial dilutions (4 μg/ml - 4 ng/ml) were prepared for each peptide-KLH conjugate. Two hundred fifty μl of each dilution were spotted 50 μl at a time onto nitrocellulose filters (Schleicher and Schuel, Keene, NH) using a Minifold filtration manifold SRC-96/0 (Scleicher and Schuell, Keene, NH). The final amounts on the nitrocellulose filter ranged from 1 μg to 1 ng. Reactivity of the antipeptide antiserum with the peptide-KLH conjugate was determined by a modification of the procedure of Eisen et al. (1988). Briefly, the filters were blocked with Tris-buffered saline (TBS; 0.14 M NaCl, 10 mM Tris, pH 8.0) containing 2% non-fat dry milk for 45 min and cut into strips. Each strip contained a set of serial dilutions for a peptide-KLH conjugate (1 μg to 1 ng) and two dilutions of KLH (1 μg and 0.1 μg). The strips were incubated overnight in 5 ml TBS containing 2% nonfat dry milk and a 1:100 dilution of the non-immune sera, immune sera, or immune sera preincubated with 100 μg/ml KLH at 4°C. Unbound antibody was removed by two 15 min washes in TBS containing 0.5% Tween 20, then 0.5 M NaCl, and finally 0.1% BSA. After washing, the strips were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Cooper Biomedical, West Chester, PA) for 2 h. The strips were then washed as above with two additional washes in TBS and incubated with 4-chloro-1 naphthol (0.6% (wt/vol); BioRad, Richmond, CA; in 1:4 methanol:TBS containing 30 μl hydrogen peroxide). The reactions were

terminated by washing in water after sufficient color development.

Cells and cell culture

The isolation and characterization of the glucocorticoid-sensitive cell line CEM-C7 was previously described (Norman and Thompson, 1977). The origins of CEM-C7 cells derivatives 6TG1.1, ICR27TK.3 and 3R7.6TG.4 were previously described (Harmon et al., 1985). Cells were grown in suspension culture in RPMI 1640 (Roswell Park Memorial Institute; Hazelton, Lexena, KS) supplemented with 10% fetal bovine serum (Hazelton, Lexena, KS) and glutamine as previously described (Norman and Thompson, 1977). Cells were maintained in logarithmic phase between 1 x 10^5 to 2 x 10^6 cells/ml. Cell number was determined using a Coulter counter (Coulter Electronics, Hialeah, FL). Ar Mor human fibroblasts (ATTC, Rockville, MD) were grown to confluency in DMEM (Dubeccos modified eagles Medium containing 4500 mg/L glucose; Hazelton, Lexena, KS) supplemented with 10% fetal bovine serum and glutamine. All of the cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of cell extracts

Cells were grown to approximately 1 x 10^6 cells/ml, harvested by centrifugation (250 x g) at 4°C for 15 min, and washed twice with 5 ml HBSS (Hanks Balanced Saline Solution; 48 mg/L Na_2PO_4, 97.7 mg/L $MgSO_4$, 60 mg/L KH_2PO_4, 400 mg/L KCl, 140 mg/L $CaCl_2$, 1 g/L glucose). The resulting cell pellet was frozen on dry ice for 20 min, then placed on wet ice to thaw. The thawed cell pellet was resuspended in Buffer 4

(10 mM HEPES, 1 mM EDTA, 10% glycerol pH 8.0) or Buffer 4 containing 20 mM sodium molybdate at a concentration of 2.5 x 10^8 cells/ml. The suspension was centrifuged at 16,000 x g for 10 min at 4°C and the supernatant (cell extract) collected. The amount of protein in the cell extract was determined by using the Bradford protein assay (Bradford, 1976; Bio-Rad, Richmond, CA) against a standard curve of bovine plasma gamma globulin (Bio-Rad, Richmond CA).

Preparation of cytosol

HTC cytosol was prepared by Paul Yen as previously described (Reichman et al., 1984). Briefly, HTC cells were washed in PBS (without calcium) and the resulting cell pellet quick frozen in dry ice for 45 min and stored at -20°C. After the pellet thawed, an equal volume of TAPS buffer (25 mM TAPS, 1 mM EDTA, 10% glycerol, pH 9.5) was added, and the solution refluxed 20X in a pipet. The resulting mixture was spun at 17,000 x g for 15 min then at 200,000 x g for 90 min. The 200,000 x g supernatant (cytosol) was collected, quick frozen, and stored in liquid nitrogen until use.

IM-9 cytosol was prepared as previously described (Harmon et al., 1984b). IM-9 cells were harvested by centrifugation (800 x g) and washed twice in HBSS. The cell pellet was resuspended in an equal volume of homogenization buffer (10 mM HEPES, 1 mM EDTA, 10 mM NaCl, 0.5 mM dithiothreitol, pH 7.6) and homogenized with 15 strokes of a Ten Broek ground glass homogenizer. The homogenate was spun at 150,000 x g for 90 min at 0-2°C. The resulting supernatant (cytosol) was adjusted to contain 10% glycerol and 0.1M NaCl, quick frozen and stored in liquid nitrogen until use.

Quantification of GR

The quantity of GR present in whole cells was determined by a modification of the whole cell binding assay previously described (Harmon and Thompson, 1981). Cells were harvested by centrifugation (250 X g) for 15 minutes at room temperature. The resulting cell pellets were resuspended in RPMI 1640 containing 20 mM HEPES (Hazelton, Lexena, KS) supplemented with 10% fetal bovine serum and glutamine at a density of 1 x 10^7 cells/ml. Nine hundred eighty microliters of the cell suspensions were incubated with 10 μl 5.0 X 10^{-6} M [^{3}H]dexamethasone (dex; 40 Ci/mmol; Amersham, Arlington Heights, Il) in the absence or presence of 10 μl of 1 X 10^{-3} M [^{1}H]dex and incubated at 37°C for 60 minutes. The total amount of radioactivity present in each sample was determined by counting a 20 μl aliquot from each sample in 2 ml Hydrofluor (National Diagnostics, Manville, NJ) in a Beckman LS 3801 Liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). At the end of the incubation, the cells were washed with 3 ml of HBSS a total of three times, and collected by centrifugation (1600 X g) for 1 minute to remove the unbound steroid. The final cell pellet was then resuspended in 1.6 ml HBSS. The amount of radioactivity remaining was determined by counting 1.0 ml of the suspension in 2 ml Hydrofluor, while cell number was determined by counting 0.2 ml of cell suspension in the Coulter Counter.

GR concentration was also determined in cell extracts. Two hundred microliters of cell extract was incubated with 2 μl of 5.0 x 10^{-6} M [^{3}H]dex in the absence or presence of 2 μl 1 x 10^{-3} M [^{1}H]dex for 2 hours at 4°C or for 30 min at 23°C. The total amount of

radioactivity in each sample was determined by counting 5 μl of each sample. At the end of the incubation, unbound steroid was removed by the addition of 50 μl of a 10% suspension of dextran-coated charcoal in Buffer 4. Dextran-coated charcoal was prepared by suspending 10 gms of activated charcoal (Sigma Chemical Co., St. Louis, MO) in 100 ml water. After decanting the fines three times, the charcoal was resuspended in 100 ml of a solution of dextran sulfate (Pharmacia, Uppsala, Sweden; 1 mg/ml) adjusted to pH 7.0 with 1N HCl and extensively dried. Samples were vortexed and spun at 2300 x g for 10 min. Fifty microliters of the supernatant from each sample were counted in 2 ml Hydrofluor in a Beckman LS 3801 Liquid scintillation counter. Specific binding was determined as the difference between the activity in samples incubated with ligand alone and those incubated in the presence of excess unlabeled ligand and expressed as fmol/mg protein. The amount of protein in each sample was determined by the Bradford protein assay (Bradford, 1976) as previously described.

The amount of GR present in each cell line as well as the apparent equilibrium dissociation constant (K_d) was also determined by Scatchard analysis (Scatchard, 1949) using whole cells as well as cell extracts. Samples were treated under the conditions described above, but with various concentrations of [^{3}H]dex.

Immunopurification of GR

Immunopurified GR from HTC and IM-9 cells were prepared by a modification of a method previously described (Smith and Harmon, 1985). HTC and IM-9 cytosols (1.0 ml) were labeled with 10 μl 1 x 10^{-5} M

[^{3}H]triamcinolone acetonide (20 Ci/mmol; Amersham, Arlington Heights, IL) in the presence of excess [^{1}H]dex or an equal volume of ethanol for 3 hours at 4°C. The labeled cytosol was divided into 250 µl aliquots and incubated at 4°C overnight with 20 µl non-immune, or immune (AC40, anti-human GR antibody; Eisen et al., 1988; or BuGR-2, anti-rat GR antibody; Gametchu and Harrison, 1984) serum. Sepharose CL-4B-immobilized Protein A (Pharmacia Fine Chemicals, Piscataway, NJ) was swollen overnight at 4°C in HEG buffer (10 mM HEPES, 1 mM EDTA, 10% glycerol pH 7.6) containing 0.5 M NaCl. After centrifugation (16,000 x g, 2 min), the resulting pellet was washed twice with 1.0 ml HEG buffer containing 0.5 M NaCl and resuspended in a final volume of 325 µl HEG buffer containing 0.5 M NaCl. Samples were adsorbed to Sepharose CL-4B-immobilized Protein A (100 µl, 30% w/vol) for 30 min followed by centrifugation at 16,000 x g for 2 min at 4°C. The resulting pellets were washed 4 times with 1.0 ml HEG buffer containing 0.5 M NaCl then twice with 1.0 ml HEG containing 0.1 M NaCl. Steroid-receptor complexes were eluted from the Sepharose CL-4B-immobilized Protein A by the addition of an equal volume of 2X Laemmli sample buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol; Laemmli, 1970) and incubation at 95°C for 5 min.

Immunoblot analysis

Immunoblot analysis was performed as previously described (Eisen et al., 1988). Proteins were fractionated by SDS-PAGE (Laemmli, 1970) on an 8% polyacrylamide gel with a 3% polyacylamide stacking gel. Molecular weights were determined from a standard curve constructed from

the mobilities of pre-stained protein standards (Bio-Rad, Richmond, CA): phosphorylase b (M_r - 101,000), BSA (M_r - 74,000), ovalbumin (M_r - 50,000), and carbonic anhydrase (M_r - 33,000). Electrophoresis was performed at constant voltage (30 V) overnight in a Hoefer SE600 series electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Directly following SDS-PAGE, proteins were electrophoretically transfered to nitrocellulose filters using a Transfor electrophoresis Unit (TE series; Hoefer Scientific Instruments, San Francisco, CA). The transfer was performed at 0.5 A for 4 hours in buffer containing 0.025 M Tris base, 0.192 M glycine and 20% methanol. Nitrocellulose filters were incubated for 1 hour with TBS containing 2% non fat dry milk then incubated overnight in 10 ml TBS containing a 1:100 dilution of AC40 or AP64. The nitrocellulose filters were then washed 2 times for 15 min with TBS containing 0.5% Tween-20, then 0.5M NaCl and finally, 0.1% BSA. The filters were then incubated with ^{125}I protein A (0.0025 μCi, 30 Ci/mg; Amersham, Arlington Heights, IL) in TBS containing 0.1% BSA for 1 hour. The filters were washed again as described above with two additional washes in TBS, air dried and exposed to film (X-OMAT AR5, Kodak, Rochester NY).

Immunoadsorption of Unactivated, Activated, and Steroid-free GR

Immunoadsorption of steroid-bound unactivated, steroid-bound activated, and steroid-free GR was performed by Dr. Paul Yen as described (Urda et al., 1989). For the immunoadsorption of HTC GR, a 60% solution of HTC cytosol in 3-{[Tris-(hydroxymethyl)methyl]amino}propanesulfonic acid (TAPS; Calbiochem, La

Jolla, CA) buffer (pH 9.5; 0°C) was incubated with 6.1-8.4 nM [^{3}H]dex or 290-350 nM [^{3}H]DM in the absence or presence of 28 μM [^{1}H]dex for 2.5 h at 4°C and diluted 1:1 with either HEG buffer containing 0.1 M NaCl or Buffer B (25 mM TAPS, 1 mM EDTA, and 10% glycerol, pH 7.6). Unactivated GR preparations also contained 20 mM sodium molybdate, whereas activated preparations were warmed for 30 min at 20°C in the absence of sodium molybdate. Steroid-free receptors were prepared under the same conditions as unactivated GR preparations, except the steroid was omitted from the initial 2.5 h incubation. Aliquots (50 μl) of each GR preparation were incubated with 10 μl pre-immune serum, AP64, BuGR-2, or Buffer B for 2 hours at 4°C, followed by the addition of 100 μl (10% wt/vol) Pansorbin (Calbiochem, La Jolla, CA) in HEG buffer containing 0.1 M NaCl. After incubation for 1 h at 4°C, adsorbed complexes were collected by centrifugation at 3160 X g and washed three times in the same buffer. Bound [^{3}H]dex was quantified by liquid scintillation counting of the pellets. [^{3}H]DM label was quantified by SDS-PAGE of 2 fold concentrated SDS extracts of Pansorbin pellets that had been washed once in the above buffer.

Sephacryl S-300 Chromatography

HTC or IM-9 cytosol (1.0 ml) was labeled with 10 μl 5 x 10^{-6} M [^{3}H]dex for 2 hr at 4°C. Unactivated preparations were labeled in the presence of sodium molybdate. Activated preparations were incubated for 30 min at 23°C. Eight hundred microliters of the unactivated or activated preparations were incubated with 200 μl of non-immune, immune AP64 or immune AP64 preincubated with Cys_{500}-Lys_{517} for 2 hrs at 4°C.

Gel filtration was performed as previously described (Eisen et al., 1986). Briefly, the 1.0 ml sample was loaded onto a 1.6 x 60 cm Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with HEG buffer containing 0.42 M NaCl and 20 M Na_2MoO_4 buffer. Columns were run at 20 ml/hr and 1.0 ml fractions were collected for measurement of radioactivity. Radioactivity was determined by counting 0.5 ml of each fraction in 2 ml Hydrofluor in a Beckman LS 3801 Liquid scintillation counter. The apparent Stokes' radius of the eluted complexes was determined from a standard curve of $(-\log K_{av})^{1/2}$ verses Stokes' radii of the globular standards thyroglobulin (R_s = 6.5 nm), ferritin (R_s = 6.1 nm), catalase (R_s = 5.2 nm), aldolase (R_s = 4.8 nm), bovine serum albumin (R_s = 3.5 nm) and hen egg ovalbumin (R_s = 3.1 nm) (Sigma, St Louis, MO). The void volume of the column was determined by the elution position of Blue Dextran 2000.

Affinity Labeling of the GR

Affinity labeling was performed as previously described (Harmon et al. 1984b) with minor modifications. Cells were harvested by centrifugation (570 x g), washed in RPMI 1640 containing 20 mM HEPES, and the cell density adjusted to 2.0 x 10^7 cell/ml. Aliquots (0.5 ml) of the cell suspensions were incubated with 2 μl of 2 x 10^{-5} M [^{3}H]dexamethasone 21-mesylate (DM; 49.9 Ci/mmol, New England Nuclear, Bostton, MA) plus 2 μl of 1 x 10^{-3} M [^{1}H]triamcinolone acetonide (TA) or 2 μl absolute ethanol for 3 hours at 4°C. Cells were washed with 1 ml HBSS and frozen in an ethanol-dry ice bath for 30 min. Samples were thawed at 23°C and resuspended in 200 μl Buffer 4. The samples were

centrifuged at 16,000 x g for 10 min, the supernatant collected, and diluted with an equal volume of 2X Laemmli sample buffer. The immunopurified proteins were fractionated by SDS-PAGE as described above. After electrophoresis, the polyacrylamide gels were stained in Coomassie Brilliant Blue R-250 and fixed with 10% acetic acid. The fixed gels were impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried using a Slab dryer (Model 483; Bio-Rad, Richmond, CA), and the radioactivity visualized by fluorography at -70°C using X-OMAT AR5 film. Molecular weights were determined from the mobilities of the ^{14}C-labeled proteins (New England Nuclear, Boston, MA): myosin (M_r = 200,000), phosphorylase b (M_r = 97,000), bovine serum albumin (M_r = 69,000) ovalbumin (M_r = 46,000), and carbonic anhydrase (M_r = 30,000).

Determination of GR activation

DNA/DEAE minicolumn assays were performed by Dr. Paul Yen as previously described (Urda et al., 1989). Briefly, DNA- and DEAE-cellulose (Pharmacia, Piscataway, NJ and Whatman, Kent, United Kingdom respectively) were washed, resuspended in Buffer B and placed in separate 1 ml tuberculin syringes (bed volume = 0.3 ml). The column containing the DNA-cellulose was placed on top of the DEAE-cellulose column, and the assembled columns were washed in 2 ml buffer B. Fifty microliters of unactivated or activated [^{3}H]dex-bound GR preparations were loaded onto the minicolumns; the columns were washed with 7 ml Buffer B and then air dried. The contents of each column were separately assayed for [^{3}H]dex radioactivity to determine the amount of [^{3}H]dex-bound GR associated with DNA- or DEAE-cellulose.

Determination of the rate of steroid dissociation (k_{off})

Cell extracts prepared in either the absence or presence of molybdate were incubated with 5 x 10^{-8} M [^{3}H]dex in the absence or presence of excess [^{1}H]dex for 2 hrs at 4°C. At the end of the incubation, additional excess [^{1}H]dex was added to all samples to prevent [^{3}H]dex reassociation. Samples were then incubated at either 4°C or 23°C. At various times, aliquots (200 μl) were taken from each tube and 50 μl of a 10% solution of dextran-coated charcoal in Buffer 4 was added to each aliquot. Samples were spun at 2300 x g for 10 min and 50 μl of the supernatant counted for radioactivity. To determine the k_{off} where dissociation appeared to be linear (ICR27, IM-9), data were expressed as ln(specific binding at time t) vs time. Linear regression was used to determine the slope (m) of the resulting straight line, and k_{off} expressed as -m. In those cases where dissociation was biphasic (6TG1.1, 3R76TG4.4), the last four time points were analyzed as above to determine the apparent k_{off} of the "slow" component. This component was subtracted from the experimental data and the k_{off} of the "fast" component determined after regression analysis of the difference.

Isolation of steroid-resistant clones

Limiting dilution (Harmon and Thompson, 1981) was used to isolate the subclone F5 from 6TG1.1. Briefly, 6TG1.1 cells were diluted in culture medium to a density of 1 cell per ml and plated in 0.2 ml portions into the wells of a 96-well microtiter tissue culture plate (Nunclon, Denmark) to ensure that no well contained more than one cell. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2

and 95% air for 10 to 14 days. At this time, the plates were microscopically examined for the presence of colonies. Well formed colonies were selected with sterile pasteur pipets and returned to 25-cm^2 tissue culture flasks (Costar, Cambridge, MA) containing 5 ml pre-equilibrated RPMI 1640 supplemented with 10% fetal bovine serum and glutamine.

Soft agar cloning was performed as previously described (Harmon et al., 1979). Several days before each experiment, a feeder layer of Ar Mor human diploid fibroblasts were seeded onto the appropriate number of 60 X 15 mm tissue culture plates (Nunclon, Denmark). Before plating the CEM-C7 cells, culture medium was removed and a 2.5 ml separator layer containing RPMI 1640, 10% fetal bovine serum, and 0.25% agarose (Type II EEO, Sigma, St. Louis, MO) $\pm$ 1 x 10^{-6} M dexamethasone at 42°C was plated directly on top of the fibroblasts. After hardening of the separator layer, one volume of cell suspension at the appropriate density (1,000 to 400,000 cells) was combined with 4.5 volumes of 2X RPMI 1640 containing 20% fetal calf serum at 37°C. To this suspension was added 4.5 volumes of 0.5% agarose at 42°C. Two and one half ml of this mixture was pipetted over the separator layer and allowed to gel at 23°C. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 10 to 14 days to allow the formation of colonies. The number of surviving cells present on each plate was determined by counting the number of colonies formed using a dissecting microscope. Colonies were selected from the agarose with a sterile pasteur pipet and returned to a 25-cm^2 tissue culture flask containing 5 ml pre-equilibrated RPMI 1640 supplemented with 10% fetal bovine serum and glutamine.

Karyotypic analysis

Chromosomal content was determined as previously described (Harmon et al., 1985). Briefly, logarithmically growing cells were exposed to colcemid (0.25 mg/ml; Gibco, Grand Island, NY) for 2-4 hours at 37°C. Cells were harvested by centrifugation (570 x g) at 23°C for 5 minutes and washed with 5 ml PBS at room temperature. The cells were swollen in 10 ml 0.075 mM KCl for 20 minutes at 23°C, collected by centrifugation (570 x g) for 2 minutes, and fixed with 3:1 methanol:acetic acid. The fixed cells were collected by centrifugation (570 x g) for 2 minutes and washed 3 times in 3:1 methanol:acetic acid. Metaphase spreads were prepared by dropping fixed cells onto chilled slides which were washed in methanol. Slides were air dried and stained with 2% Giemsa (BDH Chemicals Ltd, Poole, England) in PBS, pH 6.8, for 10 minutes. The slides were mounted using 1:1 Permount:Xylene, and photographed under a microscope (Diaplan; Leitz Wetzlar, Germany) under oil immersion. Chromosome number was determined by counting the number of chromosomes in each of 10 individual "metaphase" spreads.

Preparation of Drugs and the Dose Response Curves

Adriamycin (Sigma, St. Louis, MO) and bleomycin (Sigma, St. Louis, MO) were made as stock solutions of 128 μM and 1000 μg/ml, respectively, using sterile RPMI 1640. Chlorambucil (Sigma. St. Louis, MO) was dissolved in ethanol and diluted with three volumes of sterile RPMI 1640 to a final concentration of 5 mM. Fresh stock solutions were made for each experiment. The appropriate volume of stock solution was added to 5 ml culture flasks with cells in logarithmic phase (0.4 - 0.5

x 10^6 cells/ml). The cultures were incubated for 24 hours in the presence of the drug. After this exposure, the cells were washed free of drug and resuspended in fresh RPMI 1640 containing 10% fetal calf serum and glutamine. Cell viability after drug treatment was determined by colony formation in semi-solid agarose as described above.

Effects of dexamethasone on cell growth

Cell cultures (10 ml) were inoculated at 25,000 cells/ml and grown overnight. At this time, 10 μl of 1 x 10^{-3} M dex or 10 μl of ethanol were added to duplicate 10 ml cultures. Cell number was determined daily through the use of a Coulter counter until the cells were no longer in log phase growth. Growth curves were obtained by plotting log (cell number) versus time.

Induction of glutamine synthetase with dexamethasone

Glutamine synthetase (E.C.6.3.1.2) activity was determined by measuring the amount of gamma-glutamyl transferase activity as previously described (Harmon and Thompson, 1982). Approximately 1 X 10^7 cells were treated with 1 x 10^{-6} M dexamethasone or the same volume of ethanol for 18 to 24 hours. Cells were collected by centrifugation (250 X g) for 15 minutes at 4°C and washed twice with 5 ml RPMI 1640 without fetal bovine serum or glutamine. The resulting cell pellet was resuspended in 0.5 ml 25 mM cold sodium citrate buffer, pH 6.4, and frozen on dry ice. After thawing on ice, the cells were broken by sonication with two 10-second bursts of a sonicator (power setting 4-5; Heat Systems-Ultrasonics, Inc., Farmingdale, NY). Particulate matter

was removed by centrifugation (2300 X g) for 10 minutes at 4°C. A 0.1 ml sample of each sonicate was added to 0.3 ml of reaction mix (25 mM sodium citrate pH 6.4, 260 mM L-glutamine, 10 mM $MnCl_2$, 0.1 mM ADP, 42 mM $Na_2HAsO_4.7H_2O$, 25 mM H_2HOH) pre-heated to 37°C. Samples were vortexed and incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 0.4 ml of "stop mix" (0.2 N TCA containing 0.7 N HCl and 0.37 M $FeCl_3.6H_2O$). The samples were vortexed, particulate matter removed by centrifugation (2300 X g) for 5 minutes at 4°C, and assayed for Absorbance at 500 nm. The specific activity of the enzyme was expressed as micromoles of glutamine converted to gamma-glutamyl hydroxymate/minute/mg protein. The amount of gamma-glutamyl-hydroxymate formed was determined from a standard curve using authentic gamma-glutamyl hydroxymate (Sigma Chemical Co., St. Louis, MO).

Isolation and preparation of probes

The 402 bp GR probe (5.2 ng/μl) used for Northern blot analysis was obtained by Eco RI digestion of plasmid phGR2.9 which contains the entire hGR coding sequence (Hollenberg et al., 1985). The 18S rRNA (13 ng/μl) probe was isolated as a 1.1 kbp Eco RI/Bam HI fragment from p5B (Bowman et al., 1981). The 1.6 kbp Pst I/Cla I (8.6 ng/μl) and the 1.3 kbp Cla I/Xba I (9.8 ng/μl) fragments used for Southern blot analysis of genomic DNA were isolated from pGR107 (Hollenberg et al., 1985). Briefly, plasmid DNAs were digested with the appropriate enzyme (BRL, Gaithersburg, MD) and fractionated on 0.8-1.0% low melting agarose gels (FMC, Rockland, ME) in TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, pH 8.0) at 30 V. The fragments were excised from the gel, weighed, and

diluted with sterile water (3.0 ml/gm agarose). The amount of each fragment was calculated from total weight of the plasmid digested and the size of the insert, assuming complete digestion. The agarose/water suspension was heated to 65°C to melt the agarose and then stored at 4°.

Labeling of the probes was performed as described (Feinberg and Vogelstein, 1983, 1984). DNA (60-70 ng) was denatured at 100°C for 2 min and placed on ice. To the DNA was added 12 μl LS solution (25:25:7 1 M HEPES, pH 6.8: DTM solution: OL solution where DTM solution is 100 μM each deoxyadenosine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate in 250 mM Tris, pH 8.0; 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol; and OL solution is 1 mM Tris, pH 8.0; 1 mM EDTA, pH 7.5; and approximately 3 μg/ml hexamers), 1 μl 10 mg/ml BSA, 5 μl ^{32}p-αdCTP (3000 Ci/mmol; 10 Ci/μl, Amersham, Arlington Heights, IL), and 1 μl of the "Large Fragment" of E. coli DNA Polymerase I (Klenow fragment; BRL, Gaithersburg, MD). The mixture was incubated for several hours at 37°C, and unincorporated label present in the radiolabeled DNA was removed using G50 spin columns (5' → 3', West Chester, PA). The eluate was denatured at 100°C for 5 min before use.

Isolation of RNA

RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chromczynski and Sacchi, 1987). Briefly, cells were collected by centrifugation (520 x g, 10 min) and washed with 5 ml cold sterile PBS. The resulting cell pellet was resuspended in Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) (1 ml/10^7 cells). DNA was sheared by forcing the mixture through a 20 gauge needle 4 times. For each ml of

solution D, 0.1 ml 2 M sodium acetate, pH 4.0; 1 ml Tris (pH 8.0) saturated phenol, and 0.2 ml 49:1 chloroform:isoamyl alcohol were added. This mixture was vigorously shaken for 10 sec and incubated on ice for 15 min. Samples were centrifuged at 10,000 x g for 20 min at 4°C. The aqueous phase was removed, mixed with an equal volume of isopropanol, and the RNA precipitated overnight at -20°C. The next day samples were again centrifuged at 10,000 x g for 20 min at 4°C. RNA pellets were dissolved in 0.3 ml solution D, and precipitated with 1 volume isopropanol at -20°C for 1 hour. RNA was collected by centrifugation (16,000 x g) and washed with 75% ethanol. The resulting pellet was dryed by vacuum and then resuspended in 50 μl sterile distilled water. RNA was quantified spectrophotometrically (Gilford, Oberlin, OH) by reading A_{260}. The purity of the RNA was determined by the A_{260}/A_{280} ratio.

Northern Blot Analysis

Glyoxyl (Bio-Rad, Richmond, CA) was deionized by passage through AG501 Mixed Bed Resin (Bio-Rad, Richmond, CA) until the pH was neutral. Total RNA (5 μg) was denatured with 2 μl glyoxyl and fractionated on a 1% agarose gel in 0.01 M sodium phosphate at 100 V. RNA was transferred to Nytran filters (Schleicher and Schuel, Keene, NH) with 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) overnight and crosslinked for 2 min under the UV lamp in the tissue culture hood. The Nytran filters were prehybridized for 15 min in 10 ml Church Buffer (1% BSA, 7% SDS in 0.5 M sodium phosphate, 1 mM EDTA, pH 7.0) containing 100 μg/ml sheared denatured salmon sperm DNA. The labeled probe was denatured for 5 min

in a boiling water bath and added to 10 ml fresh Church buffer and incubated overnight with the blot at 65°C. Blots were washed twice for 15 min, under the following condititons: 10X SSC, 1% SDS at 25°C, 1X SSC, 1% SDS at 37°C, and 0.1X SSC, 1% SDS at 65°C. Blots were dried briefly and autoradiographed. When blots were to be reprobed, the hybridized probe was removed by pouring a boiling solution of 0.1% SSC 1% SDS over the blot in a glass baking dish followed by 30 min of slow cooling.

Isolation of Genomic DNA

Genomic DNA was isolated using the A.S.A.P. Genomic DNA isolation Kit (Boehringer Mannhein, Indianapolis, IN). Briefly, approximately 1 x 10^8 cells were harvested by centrifugation (120 x g) and washed with 5 ml cold sterile PBS. The pellet was resuspended in 2 ml "Lysis Buffer" containing 60 μl heat-inactivated RNase A (10 mg/ml) and incubated at 37°C for 30 min. To this mixture was added 100 μl of Proteinase K (20 mg/ml) and the mixture was incubated for 30 minutes at 60°C. After incubation, 4 ml of the "Column Wash Buffer" was added and this solution was mixed with the pre-equilibrated column matrix. The lysate-matrix mix was allowed to settle for 10 min, drain, and then washed with 3 ml "Column Wash Buffer". The column was primed with 0.5 ml "Column Elution buffer", and eluted with 2 ml "Column Elution Buffer". The DNA was precipitated by the addition of 1.2 ml isopropanol, collected by centrifugation (16,000 x g) for 20 min, and washed in 2 ml 70% ethanol. The DNA pellet was dried under vacuum and resuspended in TE buffer (10 mM Tris Cl, 1 mM EDTA) pH 7.6 at a

concentration of 1 μg/μl.

Southern blot analysis

DNA (20 μg) was digested with the appropriate enzyme (5 U/μg DNA) for 3-4 hours. Additional enzyme (5 U/μg DNA) was added, and the reaction was continued overnight. Digested DNA was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated, and washed twice in 70% ethanol. The DNA pellet was dried and resuspended in TE buffer.

Ten micrograms of digested DNA was fractionated on a 0.8% agarose gel overnight at 20V in TBE Buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 mM EDTA). The gel was immersed in 0.254 N HCl for 10 min at room temperature, rinsed with water, and then washed in denaturation solution (1.0 M NaCl, 0.5 M NaOH) two times for 15 min. Neutralization was performed in neutralization solution (0.5 M Tris pH 7.4, 1.5 M NaCl), and the DNA was transfered to Nytran filters using 20X SSC. The filters were cross-linked for 30 sec under the UV light in the tissue culture hood. Filters were hybridized and probed as described for Northern blot analysis.

RESULTS

Section I. Development and Identification of Region-specific Anti-receptor Antibodies[1]

Polyclonal antibodies were prepared against synthetic peptides corresponding to sequences of the rat GR in order to obtain region-specific glucocorticoid receptor antibodies. The peptides, Thr_{173}-Gln_{189}, Cys_{500}-Lys_{517}, Asn_{637}-Met_{652}, and Asp_{659}-Leu_{671} were conjugated to keyhole limpet hemocyanin (KLH) by Peninsula Laboratories. The peptide-KLH conjugates were used to immunize female New Zealand white rabbits. These peptides correspond to sequences Thr_{152}-Gln_{168}, Cys_{481}-Lys_{498}, Asn_{619}-Met_{634}, and Asp_{641}-Leu_{653} of the human GR, respectively. Thr_{173}-Gln_{189} is located in the amino terminal immunogenic domain, and differs from the human sequence at residue 174 (Ala → Val) (Figure 1). Cys_{500}-Lys_{517} includes the carboxyl terminal six amino acids of the DNA binding domain and the amino terminal twelve amino acids of the "hinge" region, and is completely homologous to the corresponding sequence in the human GR (Figure 2). Also located within this peptide sequence is a sequence important for nuclear localization (Picard and Yamamoto, 1987) which is homologous to sequences found in other nuclear proteins (Moreland et al., 1987; Markland et al., 1987). The last two peptide sequences are located in the carboxyl terminal steroid binding domain. Asn_{637}-Met_{652} and Asp_{659}-Leu_{671} surround Cys_{656} which has been shown to be the residue which reacts with the covalent affinity ligand dexamethasone 21-mesylate

[1]All of the work reported in this section was performed in collaboration with S. Stoney Simons and Paul Yen of the Steroid Hormones Section, NIDDK, NIH. The experiments performed by Paul Yen are noted in the text.

Figure 1. Schematic Diagram of the Rat GR and the Location of Thr_{173}-Gln_{189}.

The location of the immunogenic, DNA binding, and steroid binding domains for the rat GR are illustrated. The boundaries of each domain are based on the combined analysis of both rat and human GR (Rusconi et al., 1987; Hollenberg et al., 1987; Meisfeld et al., 1987). Chymotryptic cleavage sites are defined at residues 410 - 413 (Carlstedt-Duke et al., 1987). The peptide, Thr_{173}-Gln_{189} is located in the amino terminal immunogenic domain. The corresponding sequence in the human differs by one amino acid, an Ala ⟶ Val at residue 174.

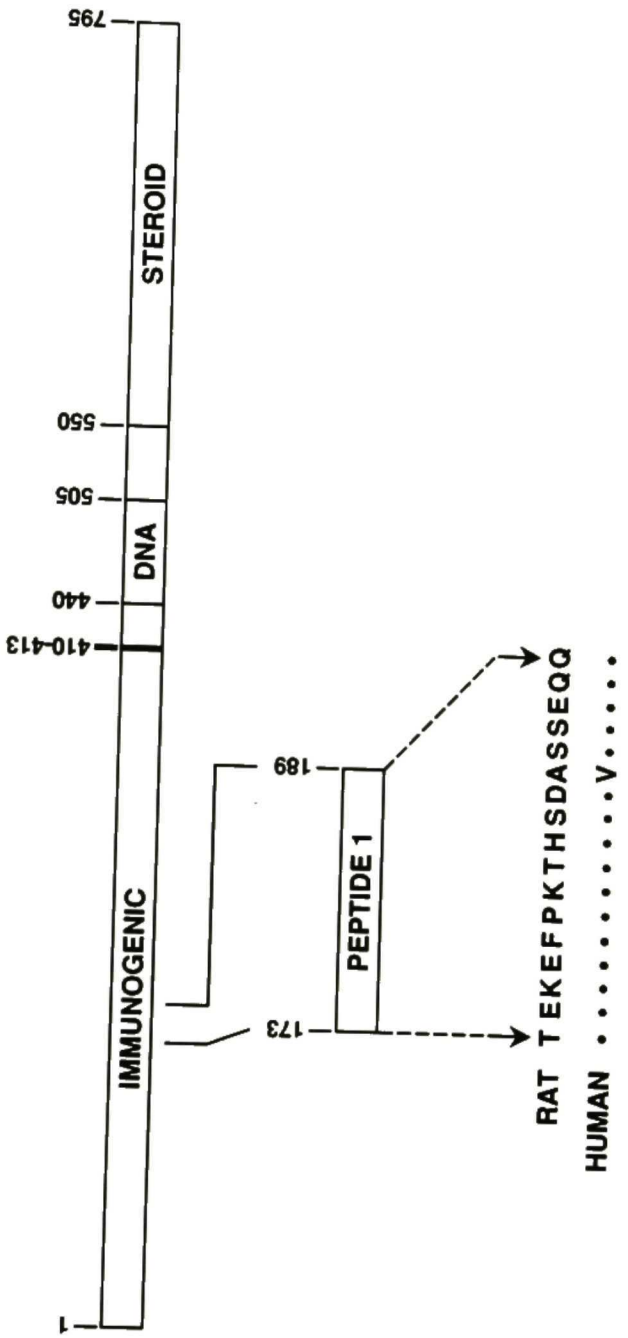
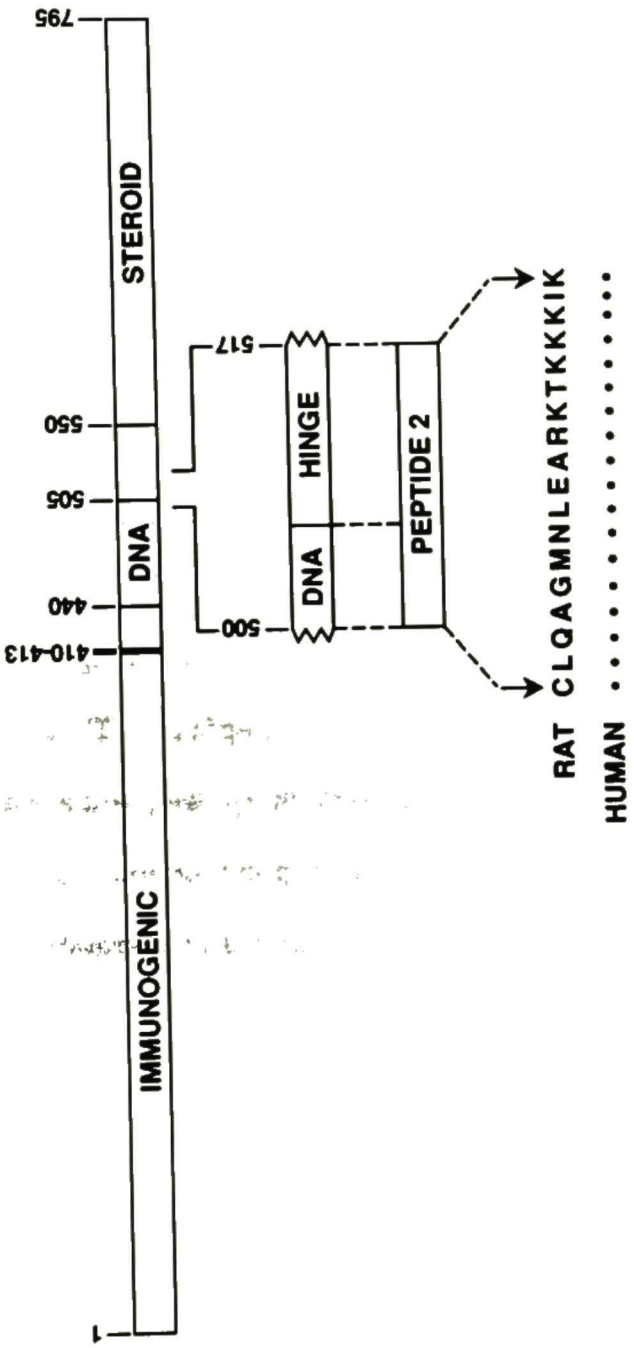

795
550
505
440
410-413
1
IMMUNOGENIC
DNA
STEROID
189
173
PEPTIDE 1
RAT T E K E F P K T H S D A S S E Q Q
HUMAN . . . . . V . . . . .

Figure 2. Schematic Diagram of the Rat GR and the Location of the Cys_{500}-Lys_{517}.

The schematic diagram illustrating the functional domains of the rat GR are described in the legend to Figure 1. Cys_{500}-Lys_{517} spans the carboxyl terminal 6 amino acids of the DNA binding domain and the amino terminal 12 amino acids of the "hinge" region. This peptide sequence is completely homologous to the corresponding sequence in the human. In addition, this peptide also contain a portion of a sequence important for nuclear localization (KKKIK) (Picard and Yamamoto, 1987).


795
STEROID
550
505
DNA
440
410-413
IMMUNOGENIC
1
517
HINGE
500
DNA
PEPTIDE 2
RAT CLQAGMNLEARKTKKKIK
HUMAN .....

(Simons et al., 1981, 1987) (Figure 3). Asn_{637}-Met_{652} is identical to the sequence in the human receptor while Asp_{659}-Leu_{671} contains a Tyr instead of a Phe at residue 666.

Two rabbits were immunized with each conjugated peptide sequence suspended in MPL + TDM emulsion, and the presence of anti-peptide-KLH conjugate antibodies was determined by dot immunoblot analysis (Figure 4). Dilutions of each peptide-KLH conjugate and KLH were spotted onto nitrocellulose filters. The reactivity to non-immune sera, immune sera, and immune sera preincubated with KLH were examined. In every case, non-immune sera failed to react with either the peptide-KLH conjugate or KLH. Immune sera reacted with the most concentrated peptide-KLH dilution and the two dilutions of KLH. Immune sera preincubated with 10^{-4} M KLH did not react with immobilized KLH, but did react with the peptide-KLH conjugate. Thus, each rabbit produced anti-peptide and/or anti-peptide-KLH antibodies.

Specificity of the anti-peptide antibody for the denatured form of the GR was determined by immunoblot analysis (Figure 5). Rat (HTC cell) and human (IM-9 cell) cytosol were immunoprecipitated with non-immune serum or the authentic anti-GR antibody AC40 (Eisen et al., 1988). Immunopurified protein was fractionated by SDS-PAGE and visualized by immunoblot analysis using non-immune (Panel A), immune serum obtained from rabbits immunized with Asp_{659}-Leu_{671} (Panel B) or AC40 (Panel C). As expected, AC40 identified a 92 kDa band characteristic of the human and rat GR (Panel C). However, non-immune serum and antibodies raised against Asp_{659}-Leu_{671} failed to react with the GR (Panels A and B). Comparable results were obtained with antibodies raised against Thr_{173}-Gln_{189}, Cys_{500}-Lys_{517}, and Asn_{637}-Met_{652}

Figure 3. Schematic Diagram of the Rat GR and the Locations of Peptides Asn_{637}-Met_{652} and Asp_{659}-Leu_{671}.

The schematic diagram of the rat GR is described in detail in Figure 1. The peptides Asn_{637}-Met_{652} and Asp_{659}-Leu_{671} are located in the steroid binding domain and surround Cys_{656} (enclosed by a square), which has been implicated in the binding of the covalent affinity ligand DM (Simons et al., 1981, 1897). Also illustrated is Met_{622} which is involved in the binding of the ligand triamcinolone acetonide (Carlstedt-Duke et al., 1988) Asn_{637}-Met_{652} is completely homologous to the corresponding sequences in the human, whereas Asp_{659}-Leu_{671} differs at residue 666 (Phe → Tyr). Both peptides, and the region of the steroid binding domain illustrated, are contained within the core steroid binding domain (Simons et al., 1989).

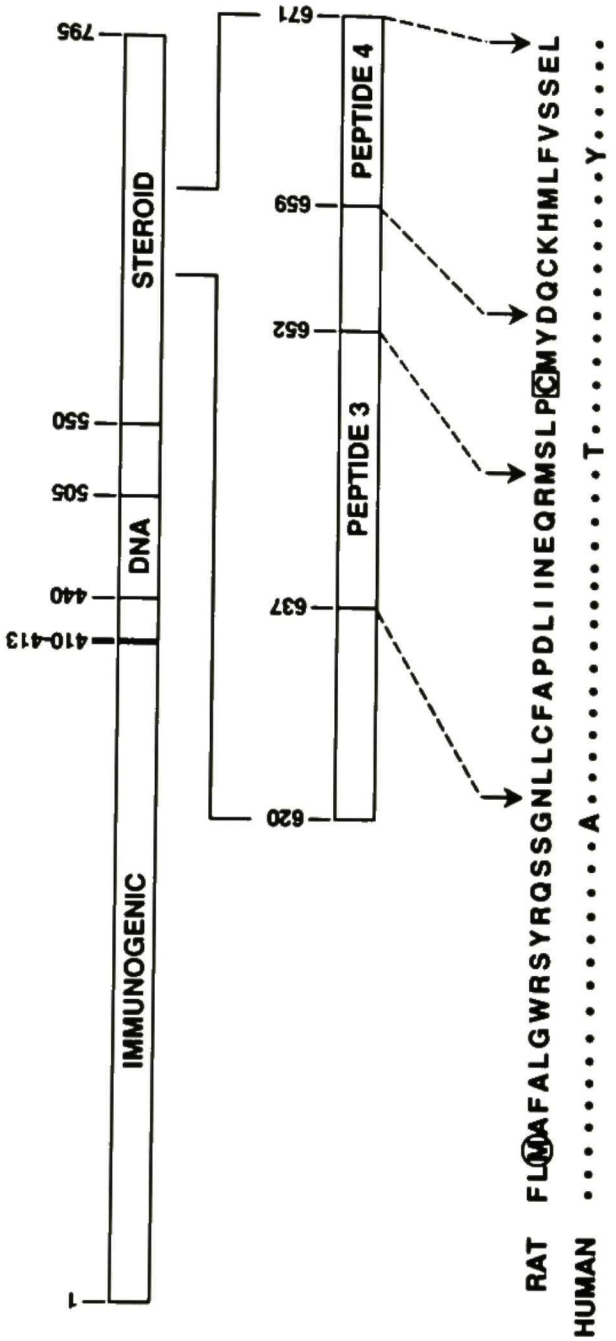

795
671
STEROID
PEPTIDE 4
659
652
550
505
DNA
PEPTIDE 3
440
637
410-413
620
1
IMMUNOGENIC
RAT FLMAFALGWRSYRQSSGNLLCFAPDLIINEQRMSLPCMYDQCKHMLFVSSEL
HUMAN .....A.....T.....Y.....

Figure 4. Dot Immunoblot Analysis of the Antisera Obtained after Immunization with the Synthetic Peptides: Thr_{173}-Gln_{189}, Cys_{500}-Lys_{517}, Asn_{637}-Met_{652}, and Asp_{659}-Leu_{671}.

The four peptide-KLH conjugates and KLH were dissolved in PBS and various amounts (μg) were spotted onto nitrocellulose filters to determine if the antiserum obtained reacted with KLH and/or the peptide conjugates as described in Methods. The reactivity of the sera with KLH and the peptide-KLH conjugates was determined by using peroxidase-conjugated goat anti rabbit IgG, 4-chloro-1-naphthol, and hydrogen peroxide.

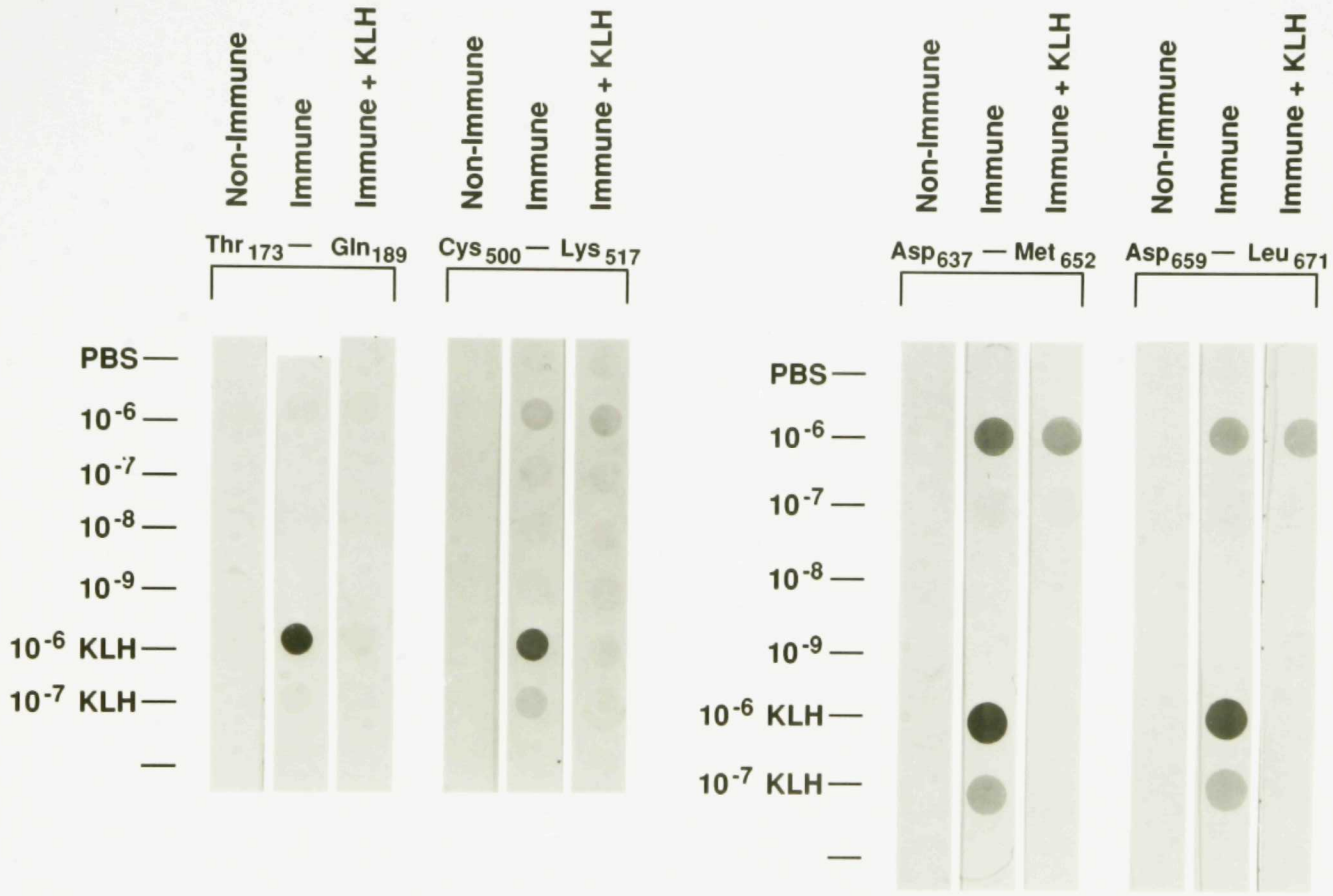
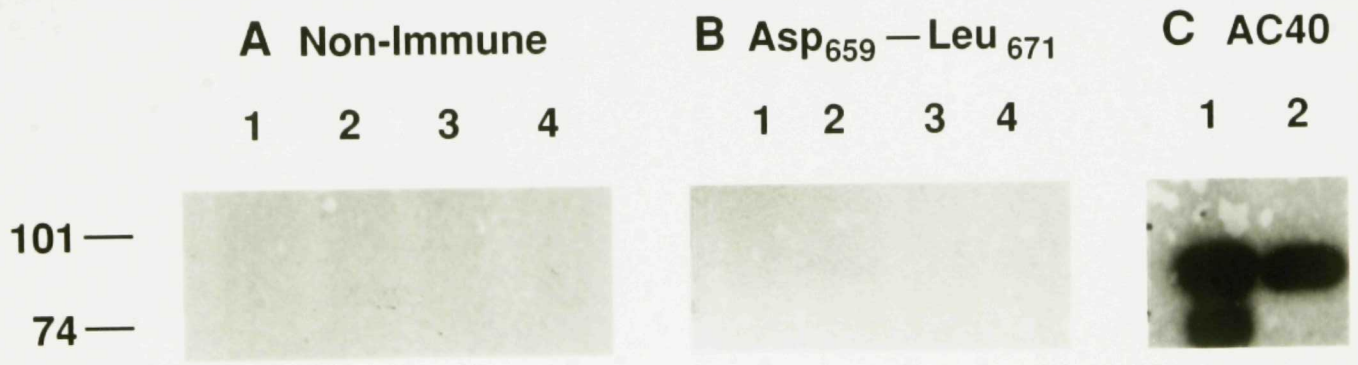

Non-Immune
Immune
Immune + KLH
Non-Immune
Immune
Immune + KLH
Thr 173 — Gln 189
Cys 500 — Lys 517
PBS
10-6
10-7
10-8
10-9
10-6 KLH
10-7 KLH
Non-Immune
Immune
Immune + KLH
Non-Immune
Immune
Immune + KLH
Asp637 — Met 652
Asp659 — Leu 671
PBS
10-6
10-7
10-8
10-9
10-6 KLH
10-7 KLH

Figure 5. Immunoblot Analysis of Human and Rat Glucocorticoid Receptors with Antireceptor Antibody AC40 and Antibodies prepared against Asp_{659}-Leu_{671}.

Panels A and B. Human IM-9 (lanes 1 and 2) and rat (HTC) (lanes 3 and 4) GR were immunopurified with either non-immune (odd lanes) or immune AC40 (even lanes) as described in Methods. The proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. The presence of immunoreactive proteins was visualized using non-immune serum (Panel A) or immune serum obtained from rabbits immunized with Asp_{659}- Leu_{671} (Panel B) and ^{125}I protein A. Panel C. Human (lane 1) and rat (lane 2) GR were immunopurified with AC40 and analyzed as in panels A and B except that AC40 antiserum was used to probe the blot. The band seen below the 92 kDa component in lane 1 represents the 78 kDa form of the IM-9 GR previously described (Harmon et al., 1984; Smith and Harmon, 1985), which appears to react preferentially with antibodies raised against the intact protein. The position of the Mr markers phosphorylase b (101 kDa) and BSA (74 kDa) are indicated.


A Non-Immune
1 2 3 4
B Asp659 — Leu 671
1 2 3 4
C AC40
1 2
101 —
74 —

(not shown).

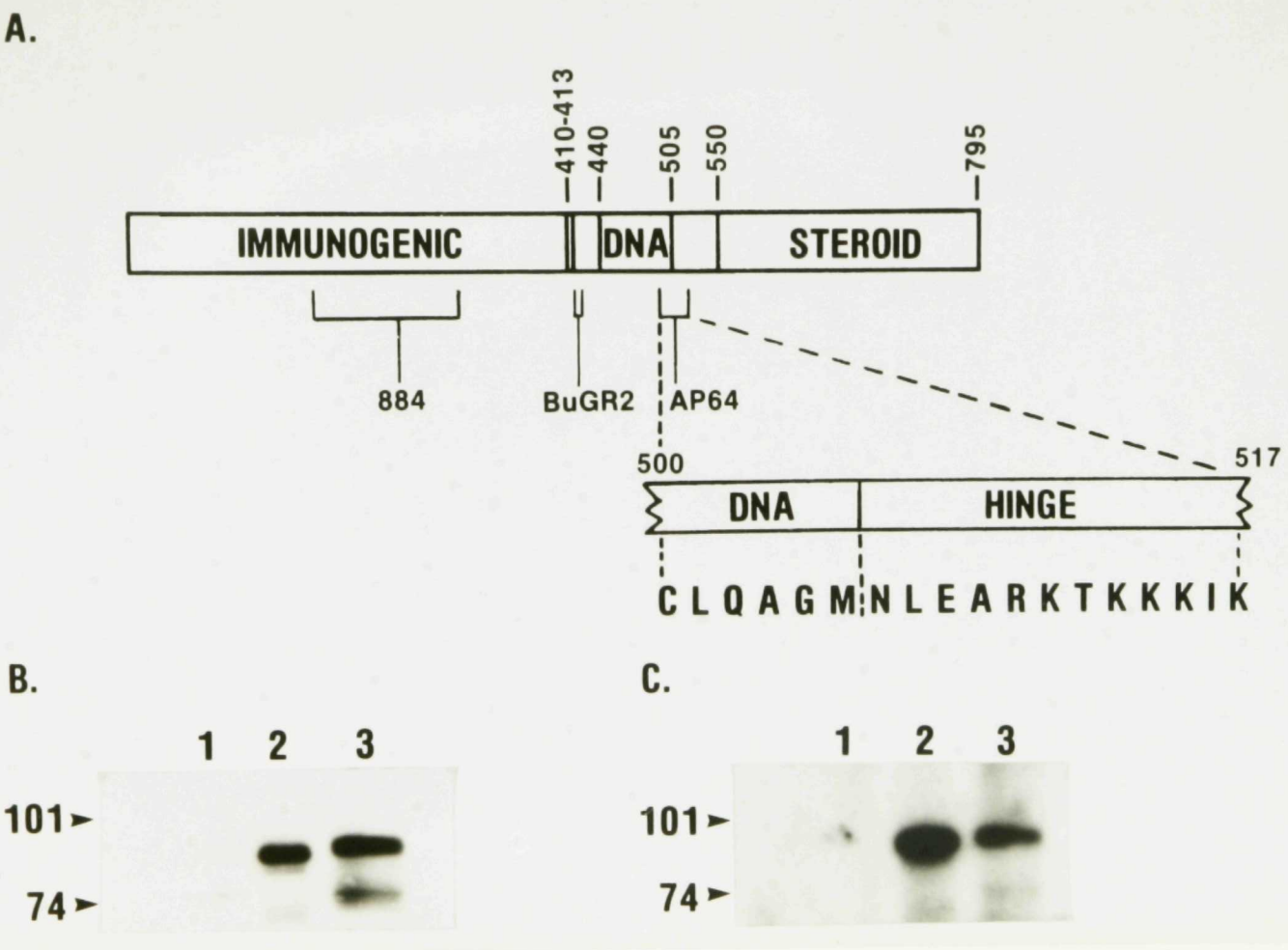
Using the above assay, no positive sera were detected two weeks after the third immunization using peptide suspended in MPL-TDM emulsion. Therefore, all rabbits were re-immunized twice with peptide-KLH conjugate suspended in Freund's Complete Adjuvant and the antisera re-evaluated for reactivity with the denatured form of the GR. Only the peptide Cys_{500}-Lys_{517} produced antibodies which reacted with the GR, and positive antisera were obtained from both immunized rabbits (AP63, AP64) (data not shown). Further analyses were performed only with the antiserum (AP64) with the higher titer. To characterize the interaction of AP64 with the denatured GR, rat (HTC cell) and human (IM-9 cell) GR were immunoprecipitated with non-immune or the known anti-GR antibodies BuGR-2 (a monoclonal anti-rat GR) and AC40 (anti-human GR) (Figure 6). The immunopurified proteins were fractionated by SDS-PAGE and visualized by immunoblot analysis using AC40 (Panel B) or AP64 (Panel C) and ^{125}I Protein A. Both AC40 and AP64 identified the same 92 kDa band characteristic of samples of rat and human GR (Lanes 2 and 3). This protein was not present after immunopurification with nonimmune serum (Lane 1). Thus, AP64 recognized the denatured form of both the rat and human GR.

In experiments performed by Dr. Paul Yen, the ability of AP64 to interact with the native GR was also determined. First, [^{3}H]dex-bound GR was incubated with pre-immune or various concentrations of immune AP64 serum and adsorbed to immobilized Protein A (Pansorbin). Serum-specific adsorption of steroid GR complexes was determined by measuring the radioactivity present in the Pansorbin pellet. A 1:25 dilution of AP64 hyperimmune sera resulted in 50% adsorption of steroid-GR complexes

Figure 6. Immunoblot Analysis of Human and Rat Glucocorticoid Receptors with Anti-receptor Antibody AC40 and Antibodies against Peptide Cys_{500}-Lys_{517}.

A. Schematic diagram of the rat GR as described in Figure 1. The locations of the antibody binding site for BuGR-2 (Gametchu and Harrison, 1984) (residues 414-423) (Rusconi et al., 1987; Eisen et al. 1985) and antibody 884 (Harmon et al., 1984; Hollenberg et al., 1985) are noted. B. IM-9 (lanes 1 and 3) or HTC (lane 2) cytosol were immunopurified with nonimmune serum (lane 1,) BuGR-2 (lane 2), or AC40 (lane 3) as described in Methods Section. After SDS-PAGE, protein was transferred to nitrocellulose, and blots were probed with AC40 and ^{125}I-protein A. Antibody AC40 was obtained from rabbits immunized with purified preparations of IM-9 cell GR and appears identical in its properties to those described for antiserum 884 (Harmon et al. 1984). C. Same as B, except that blots were probed with the anti-peptide antiserum AP64.

(From Urda et al., 1989).


A.
410-413
440
505
550
795
IMMUNOGENIC
DNA
STEROID
884
BuGR2
AP64
500
517
DNA
HINGE
C L Q A G M N L E A R K T K K K I K
B.
1 2 3
101
74
C.
1 2 3
101
74

(data not shown). Secondly, GR (HTC cell) labeled with the affinity ligand [^{3}H]DM and adsorbed to Protein A after incubation with AP64 was analyzed by SDS-PAGE and visualized by fluorography (Figure 7). A steroid binding protein of 98 kDa, identical to the size of the HTC cell GR was identified (AP -). The presence of the 98 kDa band was eliminated when the labeling reaction was performed in the presence of excess [^{1}H]dex (AP +). A small amount of the 98 kDa protein was absorbed with preimmune sera (NI), but the amount was not any different from that absorbed in the presence of buffer alone (BB). In either case, the amount present was less than seen with immune serum (AP64). Therefore, AP64 also interacts with the native GR.

The GR exists in two forms, a heteromeric, non-DNA binding (unactivated) form and a monomeric and/or homodimeric, DNA binding (activated) form. GR activation has been proposed to involve a conformational change and/or dissociation of receptor associated proteins which allow the activated GR to bind to DNA by exposing the DNA-binding domain. It is also possible that other regions of the receptor are masked in the heteromeric, non-DNA binding form. Given the fact the translocation of the GR to the nucleus is activation-dependent and the fact that AP64 was raised against a sequence shown to be necessary for nuclear localization of the GR, the ability of AP64 to interact with unactivated and activated GR was examined. To examine the ability of AP64 to interact with the "monomeric" form of the GR, [^{3}H]dex-labeled rat GR was activated and then incubated with preimmune or immune AP64 sera. The samples were analyzed by size exclusion chromatography on Sephacryl S-300. Fractions were collected and assayed for radioactivity (Figure 8, Panel A). After incubation with preimmune

Figure 7. Immunoadsorption of [^{3}H]DM-Labeled GR with AP64.

HTC cell GR was labeled with [^{3}H]DM in the absence (-) or presence (+) of an excess of [^{1}H] dexamethasone. After activation, complexes were incubated with Buffer B (BB), nonimmune serum (NI), or immune serum AP64 (AP), followed by adsorption of immune complexes onto immobilized protein-A. Adsorbed complexes were extracted, resolved by SDS-PAGE, and visualized by fluorography. The positions of the M_r markers myosin (M; M_r = 200,000), phosphorylase-b (P; M_r = 97,400), BSA (B; M_r = 66,300), ovalbumin (O; M_r = 45,000), and carbonic anhydrase (C; M_r = 30,600) are indicated.

(From Urda et al., 1989).

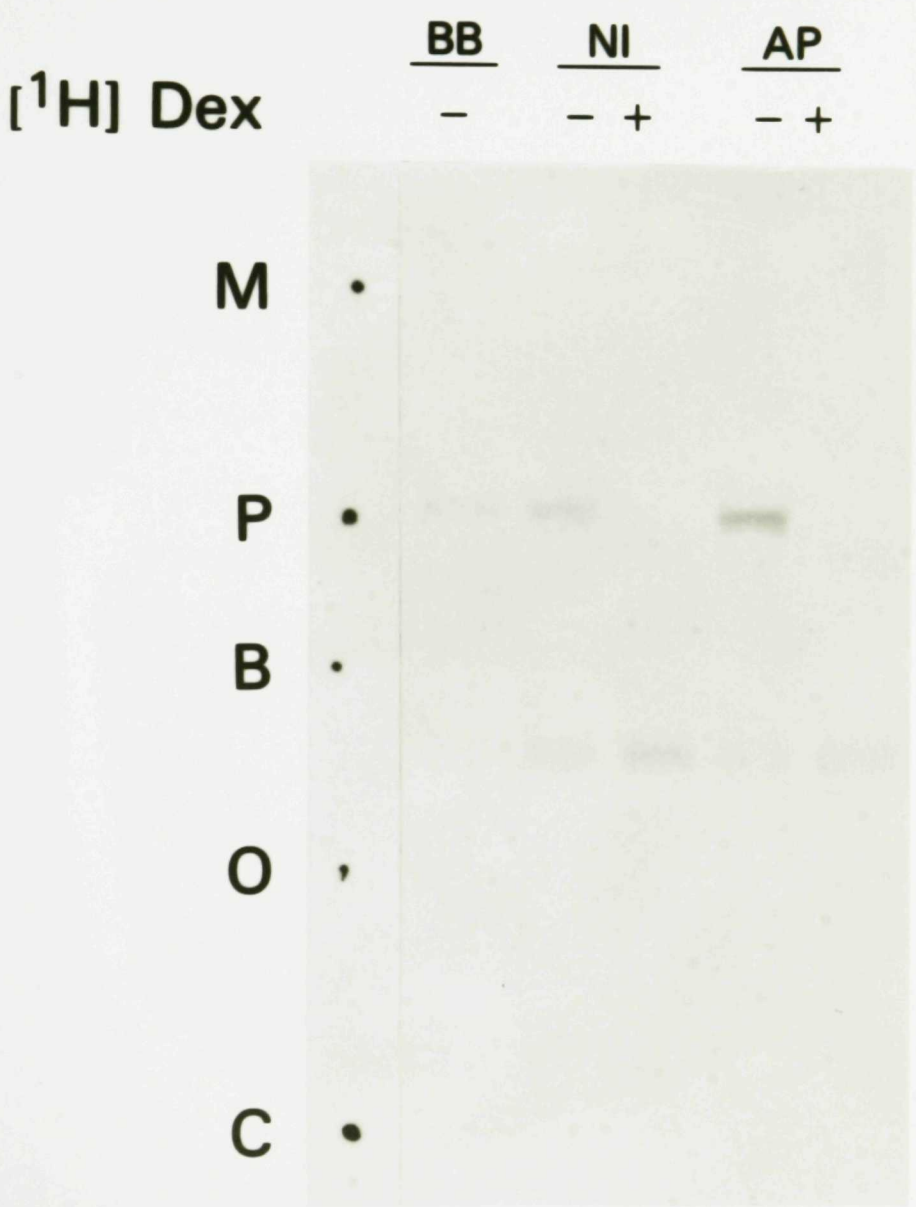
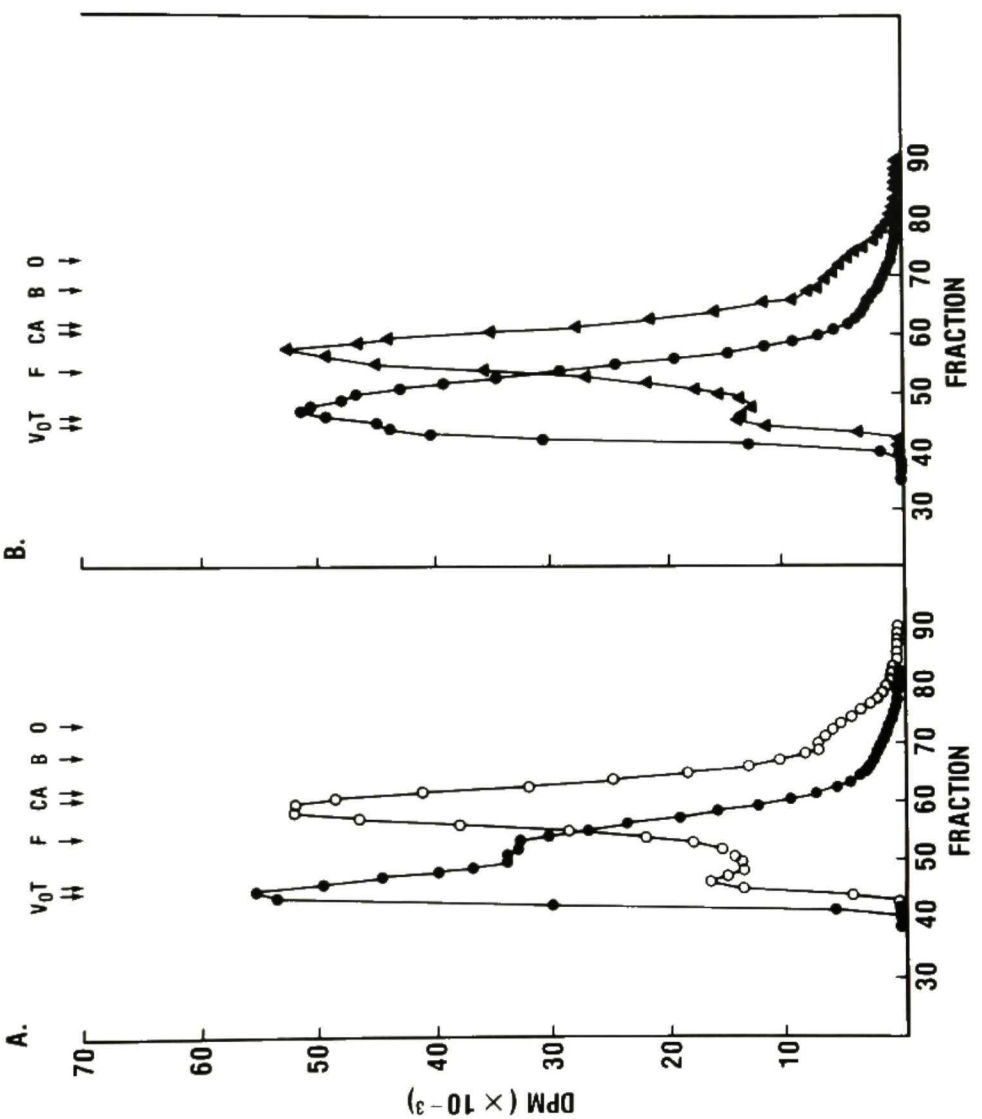

BB
NI
AP
[1H] Dex
– – + – +
M
P
B
O
C

Figure 8. Sephacryl S-300 Chromatography of Activated [^{3}H] Dexamethasone-bound Receptors after Incubation with AP64.

A. [^{3}H]Dexamethasone-bound IM-9 cell GR (800 μl) was activated at 23°C for 20 min and then incubated in the presence of 200 μl nonimmune serum (○) or immune AP64 (●) for 2 h. Samples were then chromatographed on Sephacryl S-300 in the presence of 20 mM sodium molybdate, as described in Methods Section, and 1.0 ml samples were collected and assayed for radioactivity. B. Activated GR preparations, prepared as described in A were incubated with AP64 antiserum in the absence (●) or presence (▲) of 1 μM peptide Cys_{500}-Lys_{517}. Samples were then chromatographed as described in A. Vertical arrows indicate the position of the void volume of the column (Vo) and the protein markers thyroglobulin (T), ferritin (F), catalase (C), aldolase (A), BSA (B), and ovalbumin (O). (From Urda et al., 1989).


A.
B.
V_0 T F CA B O
DPM (× 10⁻³)
FRACTION

serum (○), [^{3}H]dex-bound monomeric GR, eluted as a single 5 nm peak, the position characteristic of the monomeric GR (Eisen et al., 1986). However, in the presence of immune AP64 (●), there was a shift in the elution position toward the void volume, indicating that AP64 interacts with the GR monomer.

To assess the specificity of the antibody-receptor interaction, monomeric GR was incubated with immune AP64 in the absence (●) or presence (▲) of Cys_{500}-Lys_{517} (Figure 8, Panel B). The addition of the peptide blocked the shift in the elution position seen with AP64, indicating that the interaction between AP64 and the GR monomer is specific for the sequence Cys_{500}-Lys_{517}. In experiments performed by Paul Yen, the specificity of this interaction was confirmed by the concentration dependent ability of the peptide to block the immunoabsorption of GR by AP64 (Table 1); the GR-antibody interaction was not inhibited by the addition of the peptide Thr_{173}-Gln_{189}.

Most anti-GR antibodies made to the intact GR react with both the activated and unactivated receptor (Gametchu and Harrison, 1984; Eisen 1980; Okret et al. 1981, 1984; Harmon et al. 1984a). The latter form has been shown to be contain one steroid binding protein (Okret et al., 1985) and two molecules of hsp90 (Mendle and Orti, 1988). To determine if AP64 recognized the heteromeric form of the GR, unactivated [^{3}H]dex-labeled GR was incubated with preimmune (○) or immune (●) AP64 and subjected to Sephacryl S-300 chromatography (Figure 9). AP64 did not affect the elution position of the heteromeric GR complex, and thus does not interact with the unactivated form of the GR. This suggests that the epitope recognized by AP64 is occluded in the heteromeric form of

Table 1. Inhibition of AP64 Immunoadsorption of [^{3}H]Dexamethasone-Bound GR with Synthetic Peptides

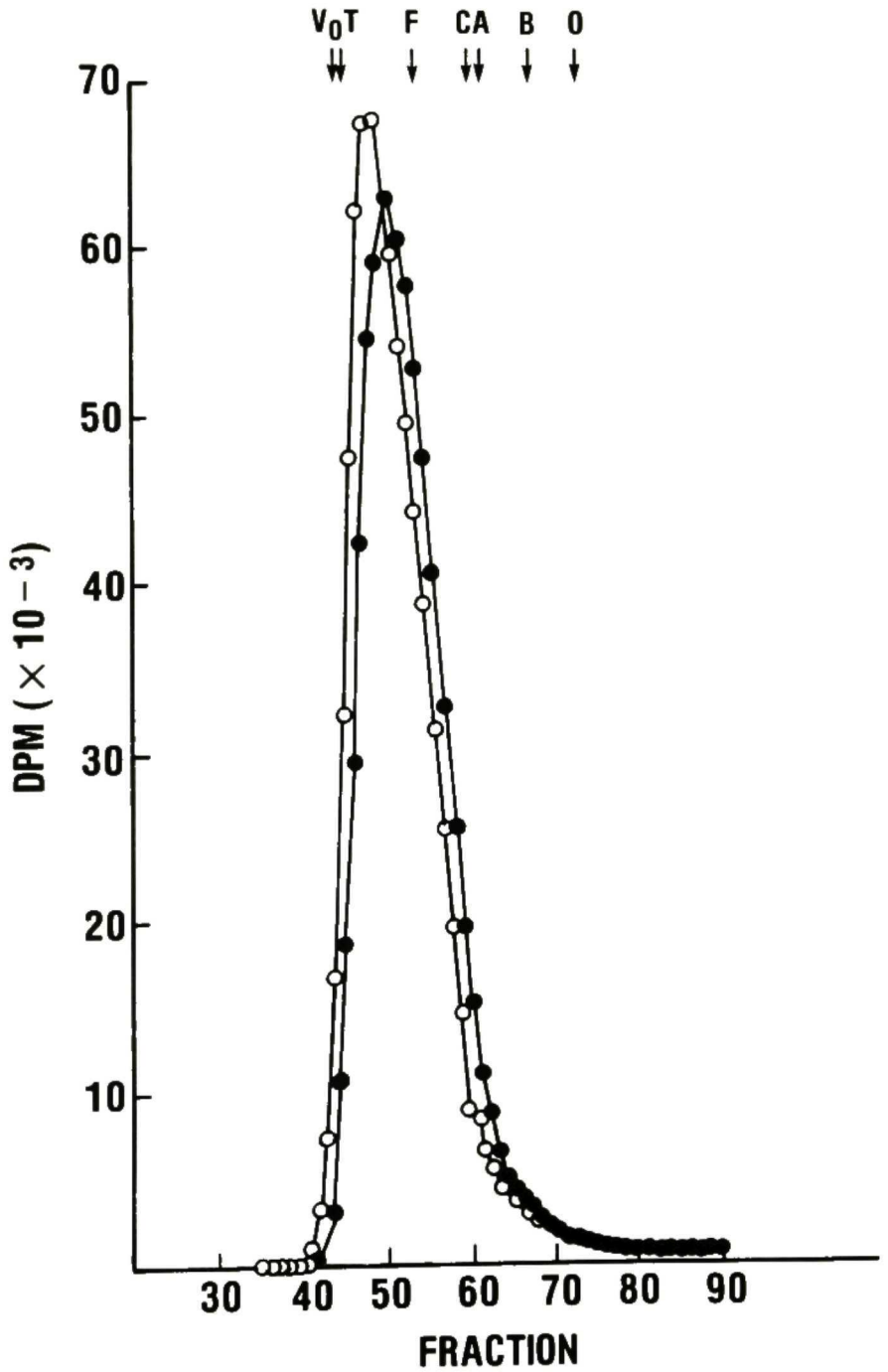
Additions		Specific ^{3}H dpm/pellet[a]	% Inhibition[b]
Serum	Peptide		
Nonimmune	None	1350	
AP64	None	8662	0
AP64	Cys_{500}-Lys_{517} (10 nM)	7814	12
AP64	Cys_{500}-Lys_{517} (100 nM)	7932	10
AP64	Cys_{500}-Lys_{517} (500 nM)	4739	54
AP64	Cys_{500}-Lys_{517} (1 μM)	1456	99
AP64	Cys_{500}-Lys_{517} (10 μM)	1329	100
AP64	Thr_{173}-Gln_{189} (10 μM)	8730	0

[a]Activated receptors were prepared as described in Methods Section. Ten microliters of serum and 10 μl peptide or Buffer B were added to 50 μl aliquots of activated receptor and immunoadsorbed as described in Methods Section.

[b]Calculated as:
$[100-(dpm_{sample} - dpm_{nonimmune})/(dpm_{AP64} - dpm_{nonimmune})]$.

(From Urda et al.,1989)

Figure 9. Sephacryl S-300 Chromatography of Unactivated [^{3}H] Dexamethasone-Bound Receptors after Incubation with AP64. Unactivated [^{3}H]dex-bound GR was incubated in the presence of 200 μl nonimmune serum (○) or immune AP64 (●) for 2 h. Samples were then chromatographed on Sephacryl S-300 in the presence of 20 mM Na_2MoO_4 as described in Figure 3.
(From Urda et al. 1989).


V_0T
F
CA
B
O
70
60
50
40
30
20
10
DPM ($\times 10^{-3}$)
30
40
50
60
70
80
90
FRACTION

the GR.

The selective interaction of AP64 with the monomeric form of the GR was also seen in the selective adsorption of activated complexes with Protein A (Table 2). In experiments performed by Paul Yen, BuGR-2 was found to adsorb unactivated and activated GR complexes with equal efficiency (57% vs. 54% respectively), while AP64 preferentially reacted with the activated complex (8.6% vs. 54%). These results were confirmed by immunoblot and fluorographic analysis of immunoadsorbed and [^{3}H]DM labeled GR (Figure 10). In experiments performed by Paul Yen, steroid-free or unactivated and activated preparations of [^{3}H]dex- (Panel A) or [^{3}H]DM- (Panels B and C) bound rat GR, were immunoadsorbed with preimmune (NI), AP64 (AP), or BuGR-2 (BU). After SDS-PAGE, samples were visualized by immunoblotting using BuGR-1 and immunoperoxidase staining (Panels A and B). AP64 failed to adsorb steroid-free or unactivated [^{3}H]dex-bound GR. On the other hand, BuGR-1 absorbed steroid-free, the unactivated and the activated [^{3}H]dex-bound GR forms. The identity of the immunoabsorbed protein as the GR was confirmed by fluorography of the [^{3}H]DM-labeled GR (Panel C).

Activated GR complexes are defined by their ability to bind to DNA. Since AP64 interacts only with the monomeric, activated GR, and the location of the immunizing peptide, Cys_{500}-Lys_{517}, contains the carboxyl terminal six amino acids of the DNA binding domain, the ability of AP64 to specifically block the binding of the activated GR complexes to DNA was examined. In experiments performed by Paul Yen, [^{3}H]dex-bound activated GR complexes were incubated with AP64 and subjected to DNA/DEAE-cellulose chromatography. Incubation with AP64 inhibited 66% of the binding of the activated GR complexes to DNA cellulose (Table 3).

Table 2. Immunoabsorption of Unactivated and Activated Preparations of [^{3}H]Dexamethasone-bound GR

Preparation	[^{3}H] dpm bound to[a]		[^{3}H] dpm immunopurified by[b]	
	DEAE-Cellulose	DNA -Cellulose	AP64	BuGR-2
Unactivated	31,235	1,407	2,829 (8.6)	18,494 (57)
Activated	9,915	14,044	12,898 (54)	12,847 (54)

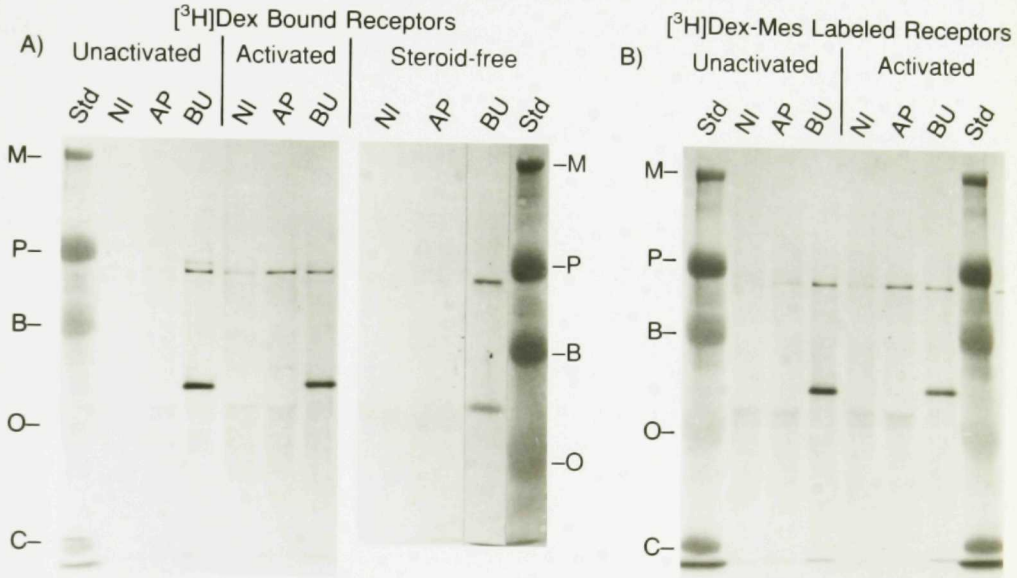
[a]Fifty microliters of HTC cytosol, bound with [^{3}H]dexamethasone as described in Methods, were subjected to coupled DNA/DEAE-cellulose chromatography as described in Methods. Column matrices were then extruded, and the disintegrations per min of radioactivity were determined.

[b]HTC cytosol was prepared as described in Methods. Ten microliters of antibody (either AP64 or BuGR-2) were added to 50 µl unactivated or activated cytosol and antigen-antibody complexes adsorbed with immobilized protein-A. The radioactivity was quantified as described in Methods. The numbers in parentheses indicate the percentage of total receptor (DNA-cellulose-bound plus DEAE-cellulose-bound) that was adsorbed.

(From Urda et al., 1989)

Figure 10. Selective Immunoadsorption of Activated Steroid-Receptor complexes by AP64.

Unactivated and activated preparations of [^{3}H]dexamethasone-bound (A) or [^{3}H]DM-labeled (B and C) HTC cell GR as well as steroid-free HTC cell GR (A) were immunoadsorbed with nonimmune serum (NI), AP64 (AP), or BuGR-2 (BU) as described in Methods. After SDS-PAGE, samples were visualized by immunoblotting using BuGR-1 and immunoperoxidase staining (A and B) or fluorography (C). For the experiment presented in C, samples were labeled with [^{3}H]DM in the absence (-) or presence (+) of an excess of [^{1}H]dex. The intense band seen at 55 kDa in A and B represents BuGR-2 heavy chain. The faint band seen above the 98 kDa band in A was not reproducibly observed. M,P,B,O,and C (defined in Figure 7) indicate the positions of the prestained protein standards, which have slightly higher apparent M_r values than the unstained standards used in Figure 10. (From Urda et al., 1989).


A)
[3H]Dex Bound Receptors
Unactivated
Activated
Steroid-free
Std
NI
AP
BU
M–
P–
B–
O–
C–
B)
[3H]Dex-Mes Labeled Receptors
Unactivated
Activated

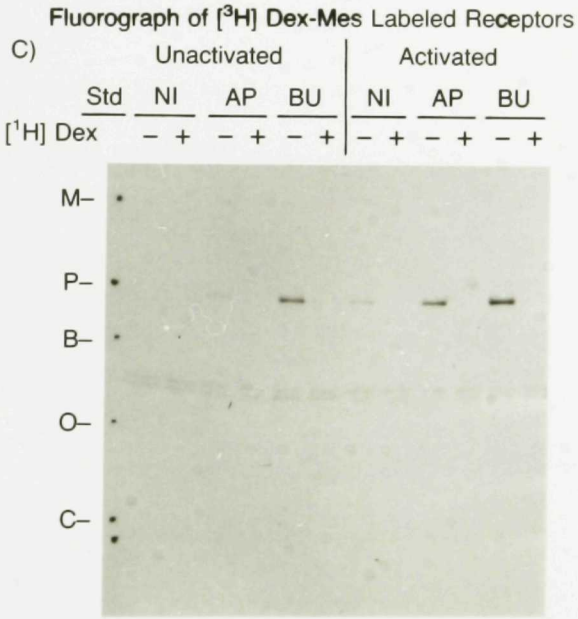

Fluorograph of [3H] Dex-Mes Labeled Receptors
C)
Unactivated
Activated
Std
NI
AP
BU
[1H] Dex
– + – + – + – + – + – +
M–
P–
B–
O–
C–

Table 3. Inhibiton of Binding of Activated Complexes to DNA-Cellulose by AP64

Additions		Specific ^3H dpm bound to DNA-cellulose[a]	% Inhibition [b]
Serum	Peptide		
Nonimmune	None	11,634	0
AP64	None	3,937	66
AP64	Cys_{500}-Lys_{517} (10 nM)	3,620	69
AP64	Cys_{500}-Lys_{517}(100 nM)	3,621	69
AP64	Cys_{500}-Lys_{517} (300 nM)	5,303	54
AP64	Cys_{500}-Lys_{517} (1 μM)	10,366	11
AP64	Cys_{500}-Lys_{517} (10 μM)	10,344	11
AP64	Thr_{173}-Gln_{189} (10 μM)	3,651	69
BuGR-2	None	14,322	0
Buffer B	None	13,121	0

[a] Activated receptors were prepared as described in Methods. Ten microliters of serum and 10 μl peptide or buffer B were added to 50 μl aliquots of activated receptor preparation and incubated for 2.5 h at 4°C. Fifty microliters of this solution were then passed over a DNA/DEAE-cellulose minicolumn, and the [^3H]dexamethsone radioactivity was quantified as described in Methods Section

[b] Calculated as $(dpm_{nonimmune} - dpm_{sample}) / (dpm_{nonimmune})$.

(From Urda et al., 1989)

This inhibition could be reversed by the addition of the peptide Cys_{500}-Lys_{517}, but not by the addition of the peptide, Thr_{173}-Gln_{189}, corresponding to a 17 amino acid sequence in the immunogenic domain. In addition, no inhibition of DNA binding activity was seen with BuGR-2. Thus, the ability of AP64 to interact with the activated GR complex and to inhibit the binding of the activated complexes to DNA-cellulose is specific for the peptide sequence Cys_{500}-Lys_{517}.

Section II. Biochemical characterization of the GR in $\underline{r}^+$ and $\underline{r}^-$ cells

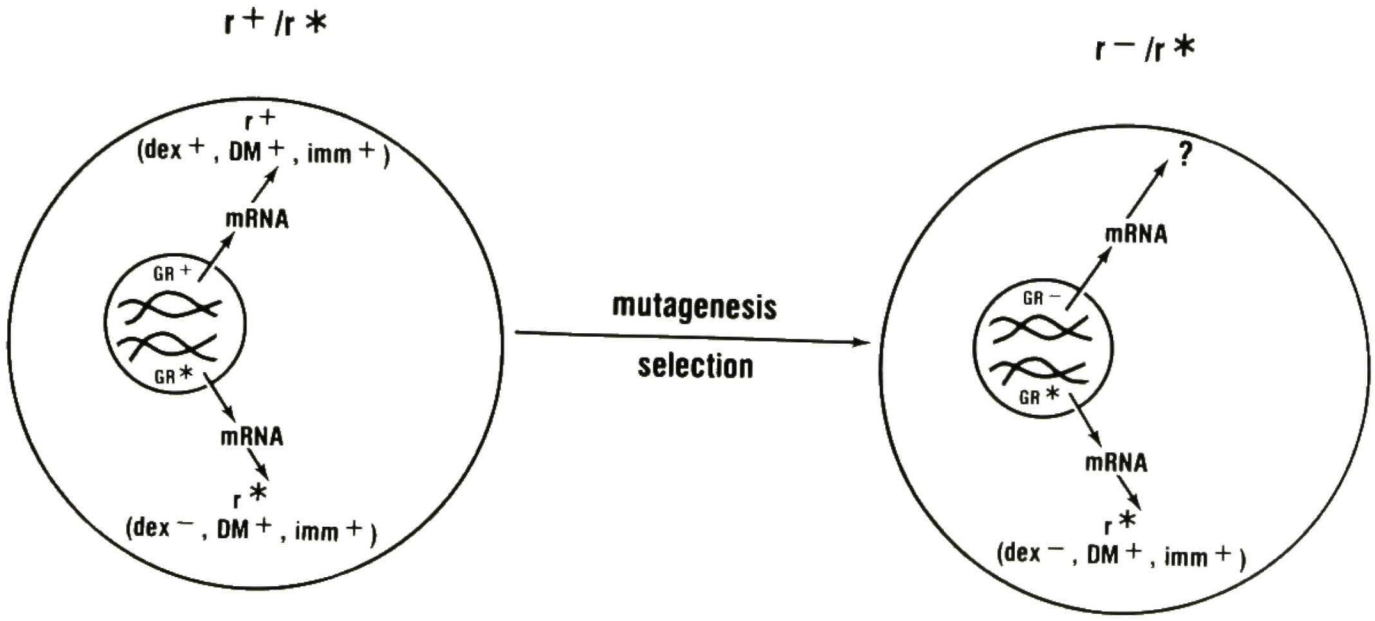
The gene which encodes the GR is autosomal (Hollenberg et al., 1985; Gehring et al., 1985). Analysis of dexamethasone-sensitive (dex^s) CEM cells (6TG1.1) and the dexamethasone-resistant (dex^r) $\underline{r}^-$ mutant ICR27TK.3 (ICR27) suggests that the genes encoding for the GR in these cell lines are dimorphic alleles (Harmon et al., 1989). One allele (GR^+) produces a functional GR protein which is able to bind steroid. The other allele (GR^*) produces a nonfunctional receptor protein which, although immunoreactive and able to bind the covalent affinity ligand DM, is unable to bind reversibly associating ligands under physiological conditions. It has been proposed that the mutational event(s) responsible for the $\underline{r}^-$ phenotype seen in ICR27 resulted in the alteration of GR^+ to GR^- such that the protein encoded by GR^- is either not produced or is rapidly degraded (Harmon et al., 1989) (Figure 11). It was therefore hypothesized that the 92 kDa GR seen in ICR27 cells is encoded by the nonfunctional GR^* allele (Harmon et al., 1989).

The dex^r cell line ICR27 was isolated after mutagenesis with the frameshift mutagen ICR 191 (Harmon and Thompson, 1981). Whole cell

Figure 11. Genotypes of dex^s $\underline{r}^+$ and dex^r $\underline{r}^-$ cells

A schematic representation of the hypothetical organization of the GR alleles in dex^s $\underline{r}^+$ cells. Each allele produces GR mRNA and GR protein. GR^+ encodes a protein that can bind dexamethasone (dex^+), dexamethasone mesylate (DM^+), and is recognized by anti-GR antibodies (imm^+). GR^* encodes a similar protein but is unable to bind dexamethasone (dex^-) under physiological conditions. The isolation of dex^r $\underline{r}^-$ cells after mutagenesis results from a mutation(s) in the GR^+ allele (GR^+ GR^-). The GR^- allele produces GR mRNA, but no GR protein. Therefore, dex^r $\underline{r}^-$ cell express only the protein encoded by GR^*.

(From Harmon et al., 1989).


r + /r *
r +
(dex + , DM + , imm +)
mRNA
GR +
GR *
mRNA
r *
(dex − , DM + , imm +)
mutagenesis
selection
r − /r *
?
mRNA
GR −
GR *
mRNA
r *
(dex − , DM + , imm +)

binding studies determined that ICR27 contained less than 10% of the binding activity seen in dex^s parental cells (Harmon and Thompson, 1981). On this basis, the cells were defined as having a "receptorless" (r^-) phenotype (Harmon et al., 1981).

To characterize the product of the GR* allele in more detail, steroid binding was examined under a variety of conditions. Whole cell binding assays performed with 6TG1.1 and ICR27 confirmed previous findings that ICR27 contained less than 10% of the binding activity seen in 6TG1.1 (Table 4). However, analysis of the steroid binding activity in cell extracts of ICR27 prepared in the absence of molybdate at 4°C showed the presence of significantly more steroid binding activity (62% of 6TG1.1) than detected in the whole cell preparations (7.9% of 6TG1.1) (Table 4). Scatchard analyses confirmed the steroid binding activity seen in the single point assays described above. In addition, they showed the presence of approximately twice as much steroid binding activity in 6TG1.1 (70 fmol/mg protein) as in ICR27 (39 fmol/mg protein), with no apparent difference in the equilibrium dissociation constants (K_d) (Figure 12, Table 5). Thus, there was significant binding activity in ICR27 when assayed at 4°C.

However, if the binding activity was measured in these cell extracts at 23°C, the steroid binding activity in ICR27 was nearly eliminated (3 fmol/mg protein), while binding activity in 6TG1.1 was only somewhat reduced (50 fmol/mg protein vs 72 fmol/mg protein) (Table 6). Thus, the GR* allele appears to encode a protein than can bind reversibly associating ligands in cell extracts at 4°C, but not at 23°C. In addition, the partial reduction in binding activity seen in 6TG1.1 extracts assayed at 23°C suggests that the protein encoded by GR* is

Table 4. Binding of [^{3}H]dexamethasone in Whole Cells and in Cell Extracts (4°C, without Sodium Molybdate) in 6TG1.1 and ICR27

Cell line	Whole cells[a] (sites/cell)	Cell extracts[b] (fmol/mg protein)
6TG1.1	20,794 ± 1860 (n=5)	72 ± 7 (n=4)
ICR27	1,650 ± 258 (n=5)	45 ± 5 (n=8)
ICR27/6TG1.1	7.9%	62%

[a] Whole cells were incubated with 5 x 10^{-8} M [^{3}H]dex in the absence or presence of excess [^{1}H]dex for 60 min at 37°C. Specific [^{3}H]dex binding was determined as described in Methods Section.

[b]Cell extracts were prepared in Buffer 4 as described in Methods Section. The extracts (200 µl) were incubated with 5 x 10^{-8} M [^{3}H]dex for 2 h at 4°C. Specific [^{3}H]dex binding was determined as described in Methods Section.

The values are the mean ± SEM for the number of experiments indicated in parenthesis.

Figure 12. Scatchard Analysis of 6TG1.1 and ICR27 in cell extracts (without molybdate) at 4°C.

Cell extracts of 6TG1.1 (●) and ICR27 (□) were prepared in Buffer 4 as described in Methods Section. Extracts (200 μl) were incubated with various concentrations of [^{3}H]dex in the absence or presence of excess of [^{1}H]dex for 2 hours at 4°C as described in Methods. Unbound steroid was removed by the addition of dextran-coated charcoal.

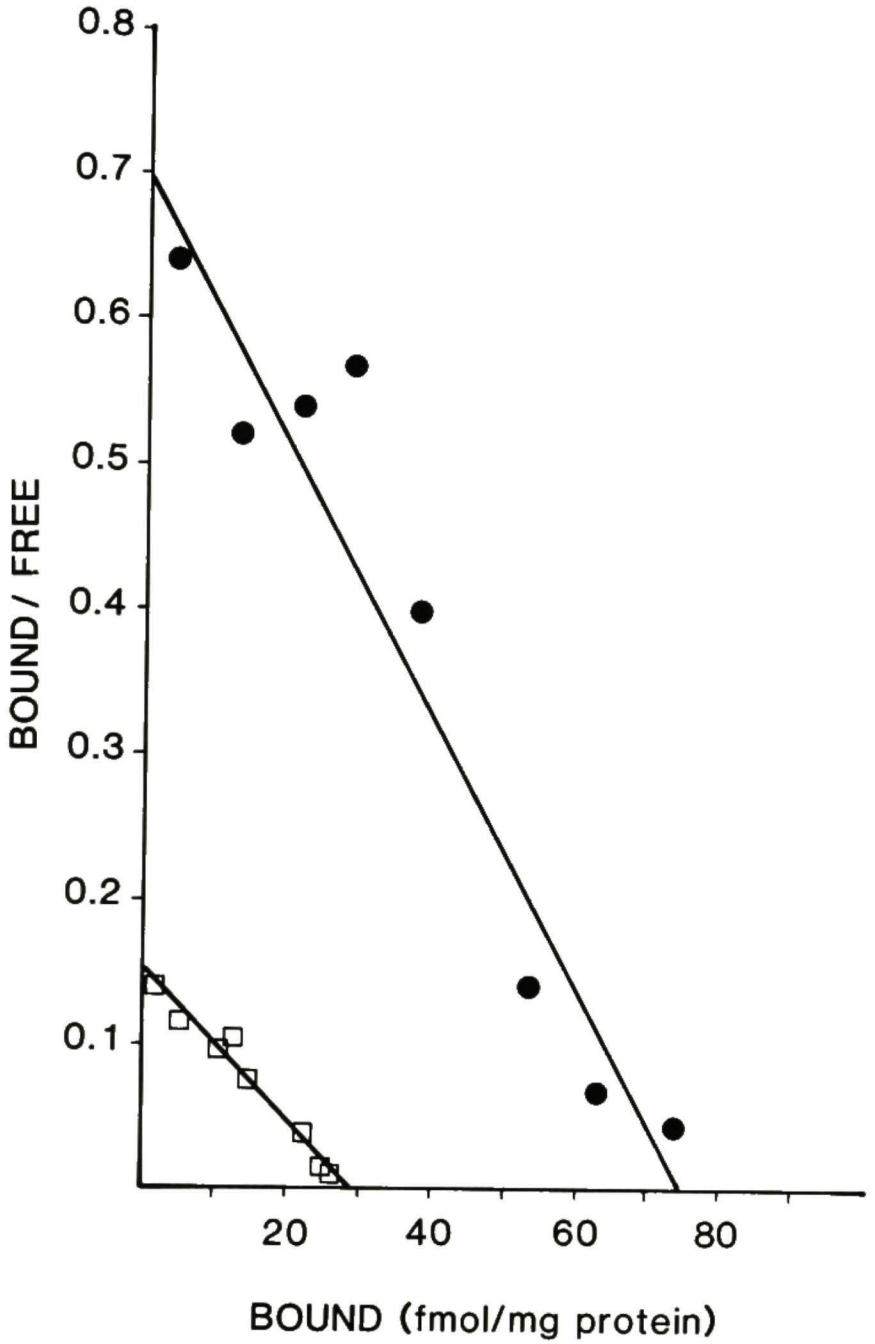

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
BOUND / FREE
20
40
60
80
BOUND (fmol/mg protein)

Table 5. Scatchard Analysis of 6TG1.1 and ICR27 in Cell Extracts (without Sodium Molybdate) at 4°C[a]

Cell line	[^{3}H]dexamethasone binding (fmol/mg protein)	K_d (M)
6TG1.1	70	4.3×10^{-9}
ICR27	39	4.7×10^{-9}

[a]The precise number of steroid binding sites and the corresponding equilibrium dissociation constants for the GR present in cell extracts of 6TG1.1 and ICR27 were determined by Scatchard analysis as described in Figure 12. The values are the average of two separate experiments.

Table 6. Binding of [^{3}H]Dexamethasone in Cell Extracts (without Sodium Molybdate) of 6TG1.1 and ICR27 at 4°C and 23°C[a]

Cell line	4°C (fmol/mg protein)	23°C (fmol/mg protein)
6TG1.1	72 ± 7 (n=4)	50 ± 2 (n=2)
ICR27	45 ± 5 (n=8)	3 ± 3 (n=3)
ICR27/6TG1.1	62%	6%

[a]Cell extracts of 6TG1.1 and ICR27 were prepared in Buffer 4 as described in Methods. Extracts (200 µl) were incubated with 5 x 10^{-8} M [^{3}H]dex in the absence or presence of excess [^{1}H] for 2 h at 4°C or 30 min at 23°C. Specific [^{3}H]dex binding was determined as described in Methods. Section. The values are the mean ± SEM for the number of experiments indicated in parenthesis.

also present in dex^s cells.

The partial loss of steroid binding activity seen in 6TG1.1, and the nearly complete loss of steroid binding activity seen in ICR27, when assayed at 23°C, suggest that the protein encoded by GR* loses its ability to bind steroid at 23°C. Alternatively, since incubation of [^{3}H]dex-labeled GR at 23°C in the absence of molybdate can result in GR activation (Schmidt and Litwack, 1982), it was considered possible that the loss of binding seen at the elevated temperature was similar to that seen in dex^r act^1 mutants. In these mutants, the instability of steroid-GR complexes during attempted activation results in the loss of steroid hormone (Schmidt et al., 1980; Harmon et al., 1984). To discriminate between these possibilities, the effect of temperature on steroid binding activity was determined in the presence of molybdate, which has been shown to block GR activation (Leach et al., 1979; Dahlmer et al., 1984) (Table 7). When binding was measured at 4°C in the presence of molybdate, the steroid binding activity seen in extracts of ICR27 was comparable to that seen in the absence of molybdate (45 fmol/mg protein verses 46 fmol/mg protein). However, in contrast to the virtual lack of binding seen when ICR27 was assayed at 23°C in the absence of molybdate, there was significant binding of [^{3}H]dex in the presence of molybdate (44 fmol/mg protein). In fact, there did not appear to be any significant difference in the binding seen in the presence of molybdate at 23°C, and that seen at 4°C in either the presence or absence of molybdate. Similarly, the presence of molybdate enhanced the binding of [^{3}H]dex in extracts of 6TG1.1 when assayed at 23°C (97 fmol/mg protein vs. 50 fmol/mg protein). However, it also appeared that molybdate enhanced the binding of [^{3}H]dex at 4°C

Table 7. Effect of Sodium Molybdate on Binding of [^{3}H]Dexamethasone in Cell Extracts of 6TG1.1 and ICR27[a]

Cell line	Temperature °C	Sodium molybdate	Binding (fmol/mg protein)
6TG1.1	4	-	72 ± 7 (n=4)
		+	119 ± 6 (n =16)
	23	-	50 ± 2 (n=2)
		+	97 ± 7 (n=4)
ICR27	4	-	45 ± 5 (n=8)
		+	46 ± 7 (n=17)
	23	-	3 ± 3 (n=3)
		+	44 ± 4 (n=17)

[a]Cell extracts of 6TG1.1 and ICR27 were prepared in Buffer 4 or Buffer 4 containing 20 mM sodium molybdate. Experiments were performed as described in Table 6. The values for the experiments performed in the absence of sodium molybdate are taken from Figure 6.

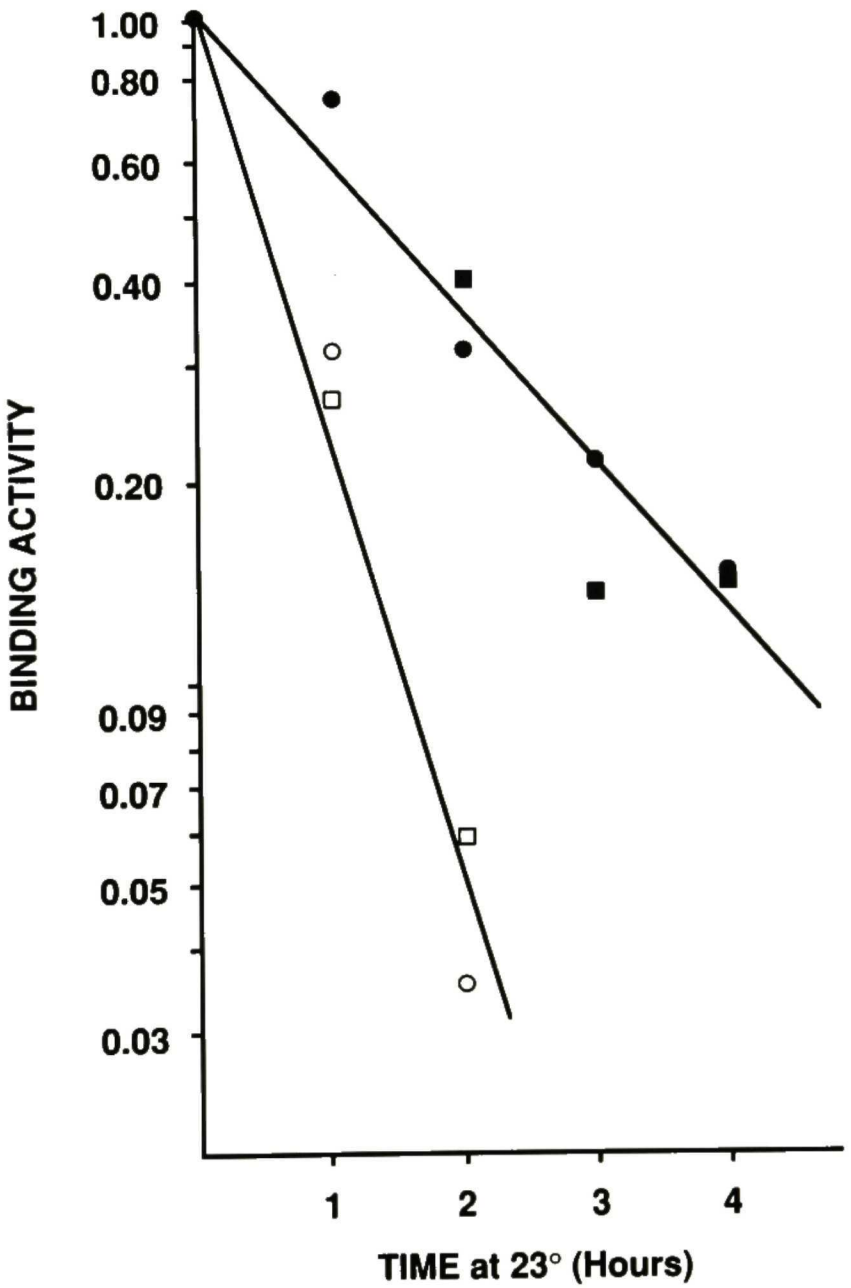
(119 fmol/mg protein vs 72 fmol/mg protein). This was contrary to the results seen in ICR27. It is not clear at this time whether this difference is the result of experimental variation or a real effect of molybdate. Regardless of the basis for increased steroid binding seen in 6TG1.1 extracts in the presence of molybdate at 4°C, the ability of molybdate to stabilize the binding of ICR27 GR at 23°C suggests that ICR27 GR does not necessarily lose its ability to bind steroid at elevated temperature.

Scatchard analyses were performed to further characterize the binding seen in ICR27 at 23°C in the presence of molybdate. The affinities of 6TG1.1 and ICR27 GR for [^{3}H]dex were indistinguishable (1.1×10^{-8} M vs 1.2×10^{-8} M) (data not shown). Consequently, the difference in the extent of binding seen in 6TG1.1 and ICR27 at 23°C in the presence of molybdate does not appear to be due to a differences in the apparent K_ds of unactivated receptors for steroid.

To further examine the effect of temperature on the integrity of the steroid binding site, cell extracts of 6TG1.1 and ICR27 were incubated at 23°C in the absence of steroid and in the absence or presence of sodium molybdate for various periods of time. Steroid binding activity was then determined at 4°C in the presence of molybdate. Extracts prepared from 6TG1.1 (circles) and ICR27 (squares) showed a time dependent decrease in steroid binding activity (Figure 13) either in the absence (open symbols) or presence (closed symbols) of molybdate. Although the presence of molybdate stabilized the ligand-free receptor in both 6TG1.1 and ICR27 (compare closed symbols with open symbols), no cell line specific differences in the rate of loss of steroid binding activity were seen. Therefore, these experiments

Figure 13. The Effects of Temperature on [^{3}H]dexamethasone binding activity in cell extracts of 6TG1.1 and ICR27.

Cell extracts of 6TG1.1 (circles) and ICR27 (squares) were prepared in Buffer 4 (open symbols) or Buffer 4 containing 20 mM sodium molybdate (closed symbols) as described in methods. The extracts were incubated in the absence of steroid for various periods of times at 23°C. Steroid binding was then determined at 4°C as described in Methods.


1.00
0.80
0.60
0.40
0.20
0.09
0.07
0.05
0.03
BINDING ACTIVITY
1
2
3
4
TIME at 23° (Hours)

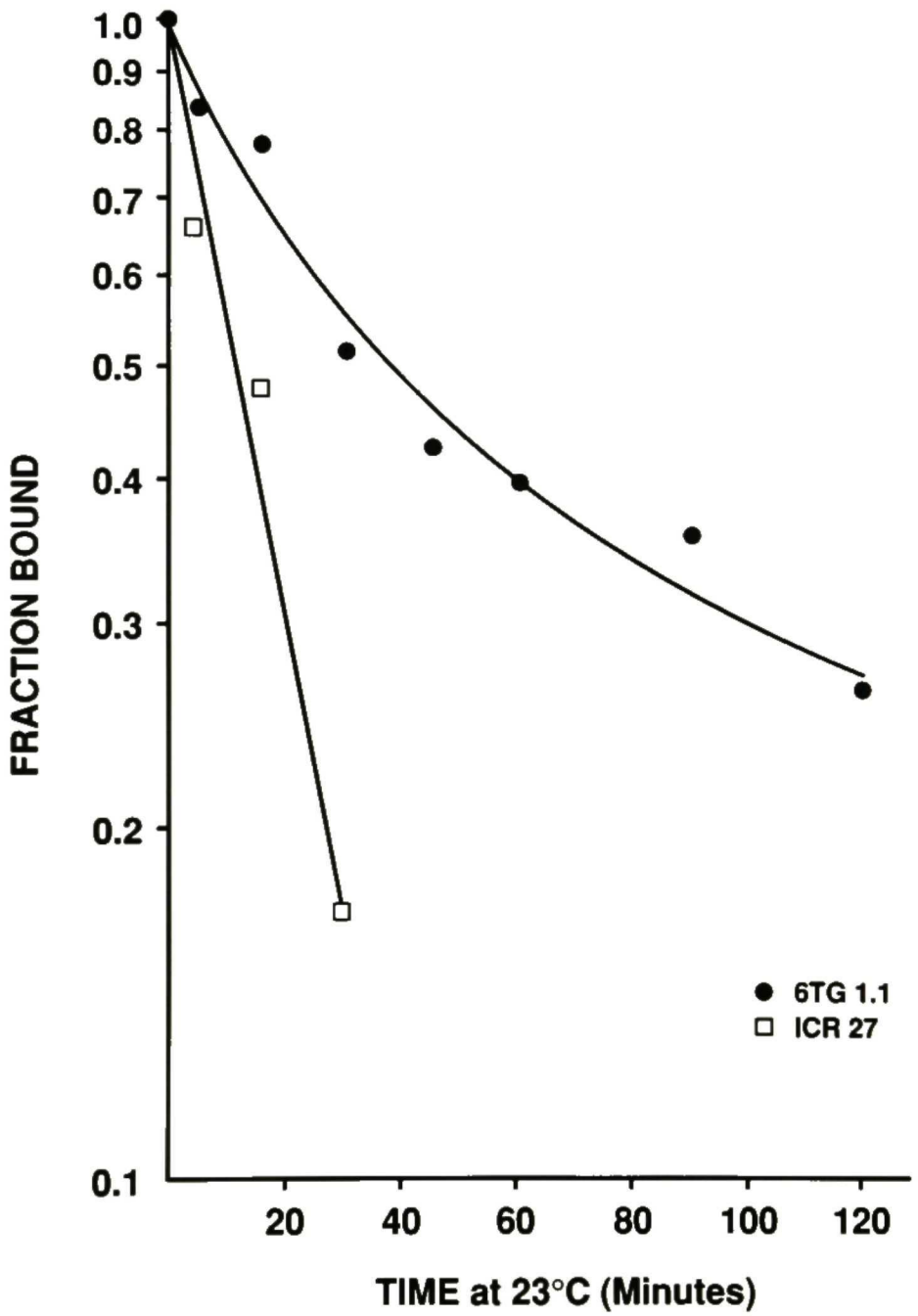
provided no evidence for a temperature-sensitive steroid-binding site in the product of the GR* allele.

The experiments described above clearly demonstrate that the binding site in the protein encoded by GR* is not temperature-sensitive. Consequently, it seemed likely that the loss of binding seen in ICR27 at 23°C in the absence of molybdate was the result of an activation-dependent process. To see if the protein encoded by the GR* allele rapidly loses steroid as a result of GR activation, a time course of steroid dissociation was performed. Extracts of 6TG1.1 and ICR27 prepared in the absence of sodium molybdate were labeled at 4°C with [^{3}H]dex in the absence or presence of excess [^{1}H]dex. After labeling, excess [^{1}H]dex was added to all samples to prevent reassociation of the [^{3}H]dex. Samples were then placed at 23°C to promote activation. Aliquots were taken at various time points and assayed to measure the presence of bound steroid. The results showed that the rate of dissociation of steroid from ICR27 receptors was significantly faster than the rate of dissociation from 6TG1.1 (Figure 14). This result is consistent with the hypothesis that the GR* allele encodes an <u>act</u>^{1} or <u>act</u>^{1}-like protein.

However, inspection of the dissociation curves in Figure 14 revealed that while dissociation from ICR27 was linear (k_{off} = 0.056/min), the dissociation curve obtained from 6TG1.1 was curvilinear. Further analysis of the dissociation curve from 6TG1.1 identified the presence of two components, a slow component with a k_{off} = 0.0070/min and a fast component with a k_{off} = 0.032/min. Such a curvilinear curve is consistent with the observation that dissociation of steroid from activated receptor is slower than the dissociation of

Figure 14. Steroid Dissociation Curves for 6TG1.1 and ICR27 at 23°C without molybdate

Cell extracts of 6TG1.1 (●) and ICR27 (□) were prepared in Buffer 4 as described in Methods Section. Extracts were incubated with 5 x 10^{-8}M [^{3}H]dex in the absence or presence of 1 x 10^{-5} M [^{1}H]dex for 2 hours at 4°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. The samples were placed at 23°C and aliquots (200 μl) were taken after various times at 23°C and analyzed for steroid binding activity. Unbound steroid was removed by the addition of dextran-coated charcoal.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FRACTION BOUND
20
40
60
80
100
120
TIME at 23°C (Minutes)
6TG 1.1
ICR 27

steroid from unactivated receptors (Nemoto et al., 1989).

Based on the experiments described above, if the only defect in the protein encoded by the GR* allele were an act^1 or act^1-like mutation, then we would predict that in the presence of molybdate: 1) dissociation experiments would show a monophasic dissociation curve for 6TG1.1; and 2) the rates of steroid dissociation from 6TG1.1 and ICR27 should be identical. To test these predictions, time courses of steroid dissociation were performed in the presence of molybdate. Surprisingly, the dissociation curve from 6TG1.1 was still biphasic and the dissociation rate from ICR27 was extremely rapid (k_{off} = 0.037/min) (Figure 15, Table 8). These results cannot be attributed to GR proteolysis since immunochemical analysis of samples taken during the time course of dissociation above showed there is no decrease in the amount of immunoreactive 92 kDa GR protein present in the cell extracts with time in either 6TG1.1 (Figure 16, Panel A) or ICR27 (Figure 16, Panel B). Nor can these results be attributed to GR activation since analysis of the receptors by DEAE- and DNA-cellulose chromatography indicated no significant receptor activation (Table 9). In addition, when dissociation was measured at 4°C, comparable results were obtained (Figure 17, Table 10). Thus, it appears that while the protein encoded by the GR* allele contains an act^1 or act^1-like mutation, there is an additional defect which increases the rate of steroid dissociation from the unactivated form of the GR. Indeed, when the rate of steroid dissociation was measured from an authentic act^1 mutant (3R7) at 23°C in the presence of molybdate, a curvilinear dissociation curve containing both a slow (k_{off} = 0.0078/min) and a fast (k_{off} = 0.024/min) component was seen (Figure 18).

Figure 15. Steroid dissociation curves for 6TG1.1 and ICR27 at 23°C in the presence of 20 mM sodium molybdate

Cell extracts of 6TG1.1 (●) and ICR27 (□) were prepared in Buffer 4 containing 20 mM sodium molybdate as described in Methods. Cell extracts were incubated with 5 x 10^{-8} M [^{3}H] dex in the absence or presence of 1 x 10^{-5}M [^{1}H]dex for 2 hours at 4°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. Samples were placed at 23°C and aliquots were taken after various times of incubation at 23°C and analyzed for steroid binding activity. Unbound steroid was removed by the addition of dextran coated charcoal. The values represent the mean ± SEM for 4 experiments.

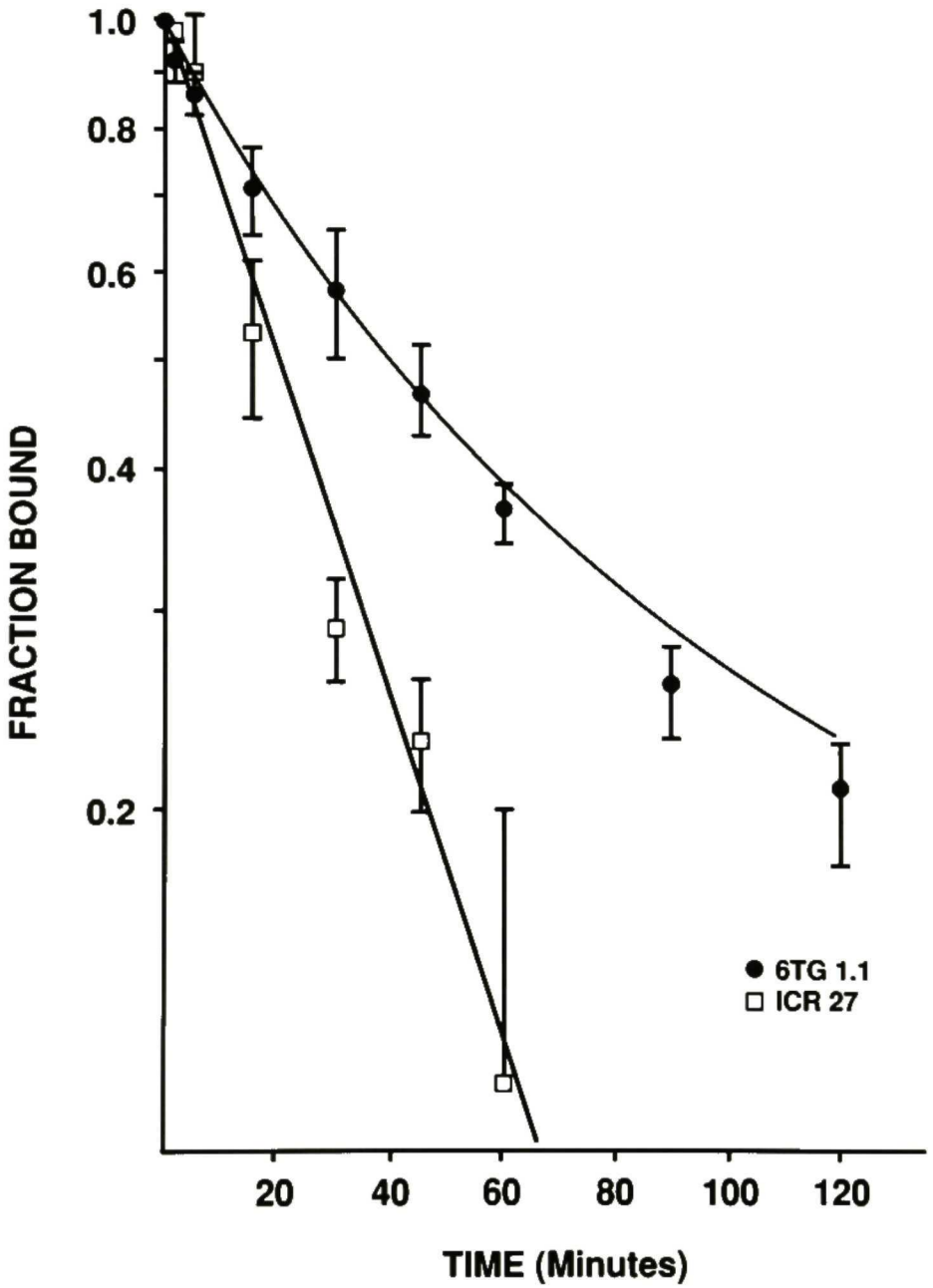

1.0
0.8
0.6
0.4
0.2
FRACTION BOUND
20
40
60
80
100
120
TIME (Minutes)
6TG 1.1
ICR 27

Table 8. Rates of [^{3}H]Dexamethasone Dissociation (k_{off}) for 6TG1.1 and ICR27 at 23°C (with Sodium Molybdate)[a]

Cell line	k_{off} (min $^{-1}$)		Half life (min)	
	k_1	k_2	t_1	t_2
6TG1.1	0.0066	0.0391	104	17.7
ICR27		0.0370		18.7

[a]The above table summarizes the values obtained from Figure 15. The values are the average of 5 individual experiments. The k_{off} of each component of 6TG1.1 was determined as described in Methods Section.

Figure 16. Immunoblot analysis of the time course of 6TG1.1 and ICR27 at 23°C in the presence of sodium molybdate

A. Samples taken from the time course of steroid dissociation of 6TG1.1 at 23°C in the presence of 20 mM sodium molybdate were analyzed for the presence of GR protein. Samples were fractionated by SDS-PAGE and immunoblotted. The immunoreactive protein was visualized by using the anti-GR antibody AC40 and ^{125}I-Protein A.

B. Same as A, but with samples obtained from the time course of steroid dissociation of ICR27 at 23°C in the presence of 20 mM sodium molybdate.

C. [^{3}H]Dexamethasone mesylate labeling of the GR in clone F5. Cells were incubated with the covalent affinity ligand [^{3}H]dexamethasone mesylate in the absence (U) or presence (C) of excess triamcinolone acetonide as described in Methods. GR was visualized by fluorography.

D. Immunopurification of CEM cell GR. GR from clone F5 was immunopurified with either non-immune or immune AC40, fractionated by SDS PAGE and transferred to nitrocellulose as described in Methods. The immunoreactive protein were visualized by AC40 and ^{125}I-Protein A. AC40 identifies a 92 kDa band as the GR.

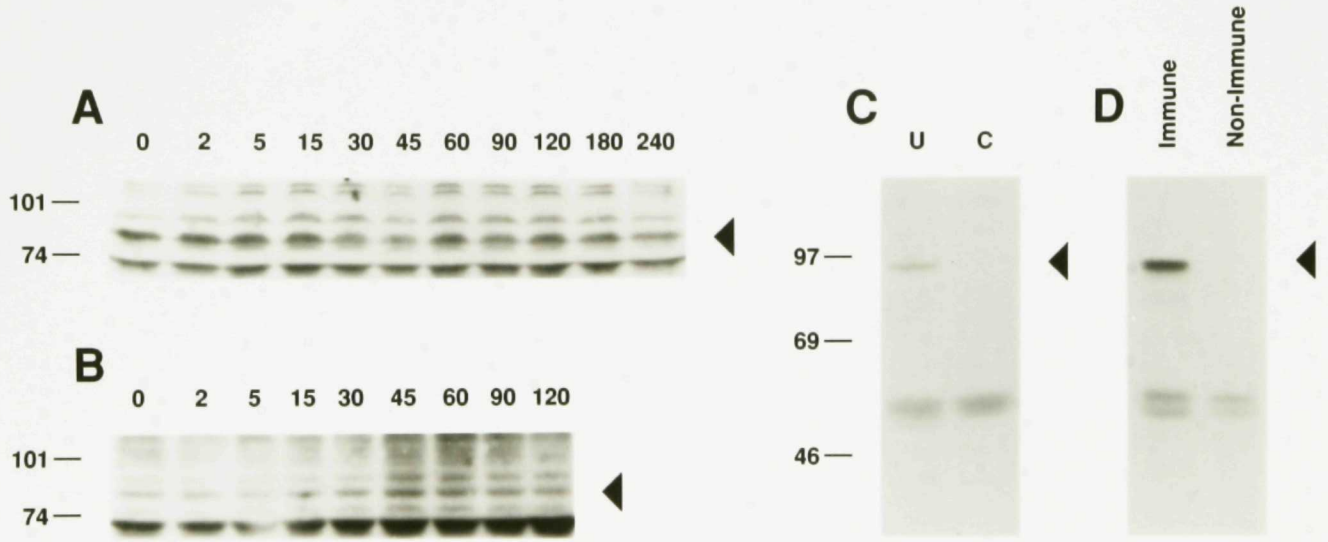

A
0 2 5 15 30 45 60 90 120 180 240
101
74
B
0 2 5 15 30 45 60 90 120
101
74
C
U C
97
69
46
D
Immune
Non-Immune

Table 9. Presence of Activated [^{3}H]Dexamethasone-Bound GR in 6TG1.1 and ICR27[a]

Cell line	dpm bound to DNA -cellulose	dpm bound to DEAE-cellulose	Time at 23°C	% Bound to DNA[b]
6TG1.1	267	5525	0	4.6
	193	1886	90	9.3
ICR27	87	3117	0	2.7
	91	1056	30	7.9

[a]During the time course of steroid dissociation at 23°C in the presence of sodium molybdate, samples (100 µl) were collected and examined for their ability to bind to DNA- or DEAE- cellulose. The DNA and DEAE-cellulose columns were washed three times with 1.0 ml HEG buffer. Fifty µl of the sample were applied to each column, the columns were washed twice with 1.0 ml HEGM buffer and the radioactivity eluted with 1.0 ml HEG buffer containing 0.5 M NaCl. The reduced amount of radioactivity seen in 6TG1.1 at 90 min and in ICR27 at 30 min reflect the amount of steroid binding activity remaining in each sample at the corresponding time points during the dissociation experiments.

[b]Calculated as: $dpm_{DNA\text{-}cellulose}$/ $dpm_{DEAE\text{-}cellulose}$.

Figure 17. Steroid dissociation curve for 6TG1.1 and ICR27 at 4°C in the presence of molybdate.

Cell extracts of 6TG1.1 (●) and ICR27 (□) were prepared in Buffer 4 containing 20 mM sodium molybdate as described in the Methods Section. Cell extracts were incubated with 5 x 10^{-8} M [^{3}H]dex in the absence or presence of 1 x 10^{-5}M [^{1}H]dex for 2 hours at 4°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. Samples remained at 4°C and aliquots were taken after various times of incubation at 4°C and analyzed for steroid binding activity. Unbound steroid was removed by the addition of dextran-coated charcoal.

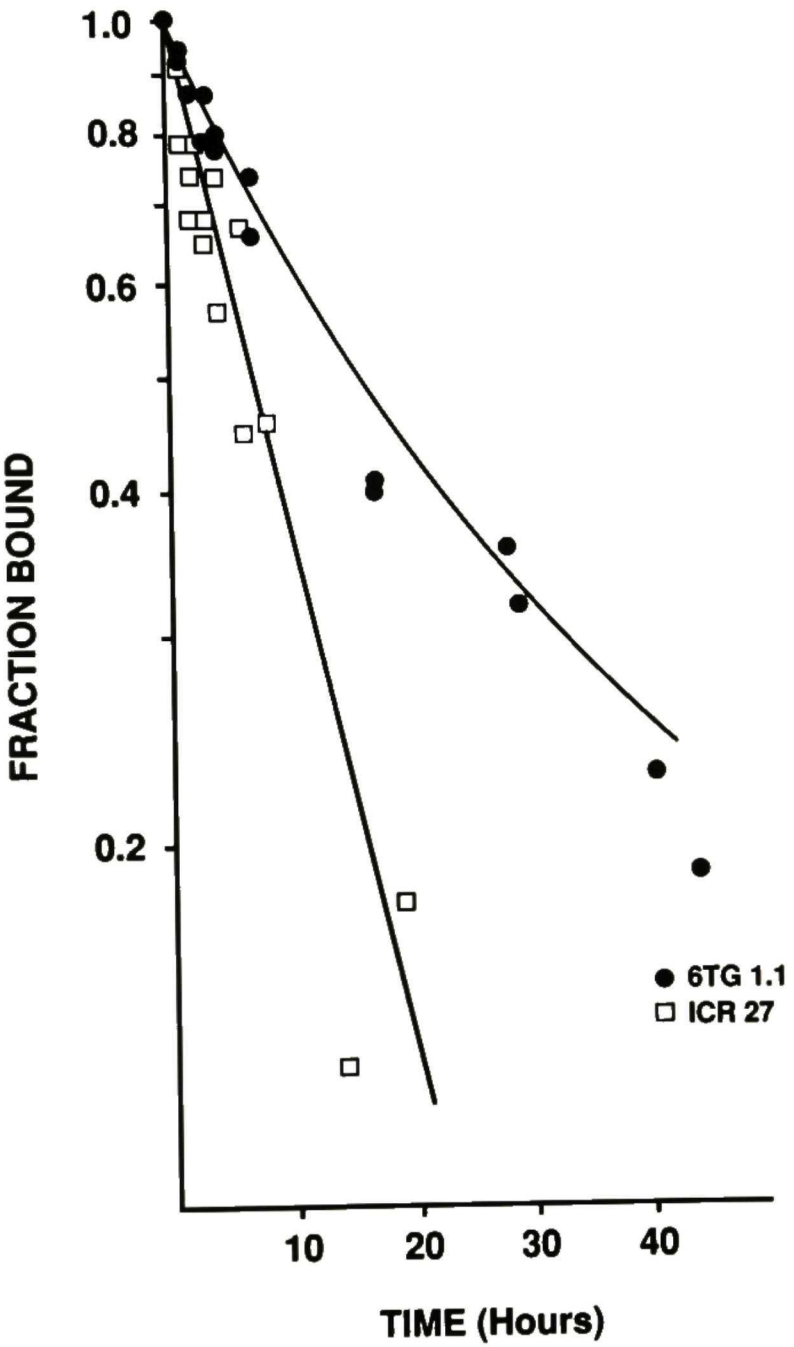

1.0
0.8
0.6
0.4
0.2
FRACTION BOUND
10
20
30
40
TIME (Hours)
6TG 1.1
ICR 27

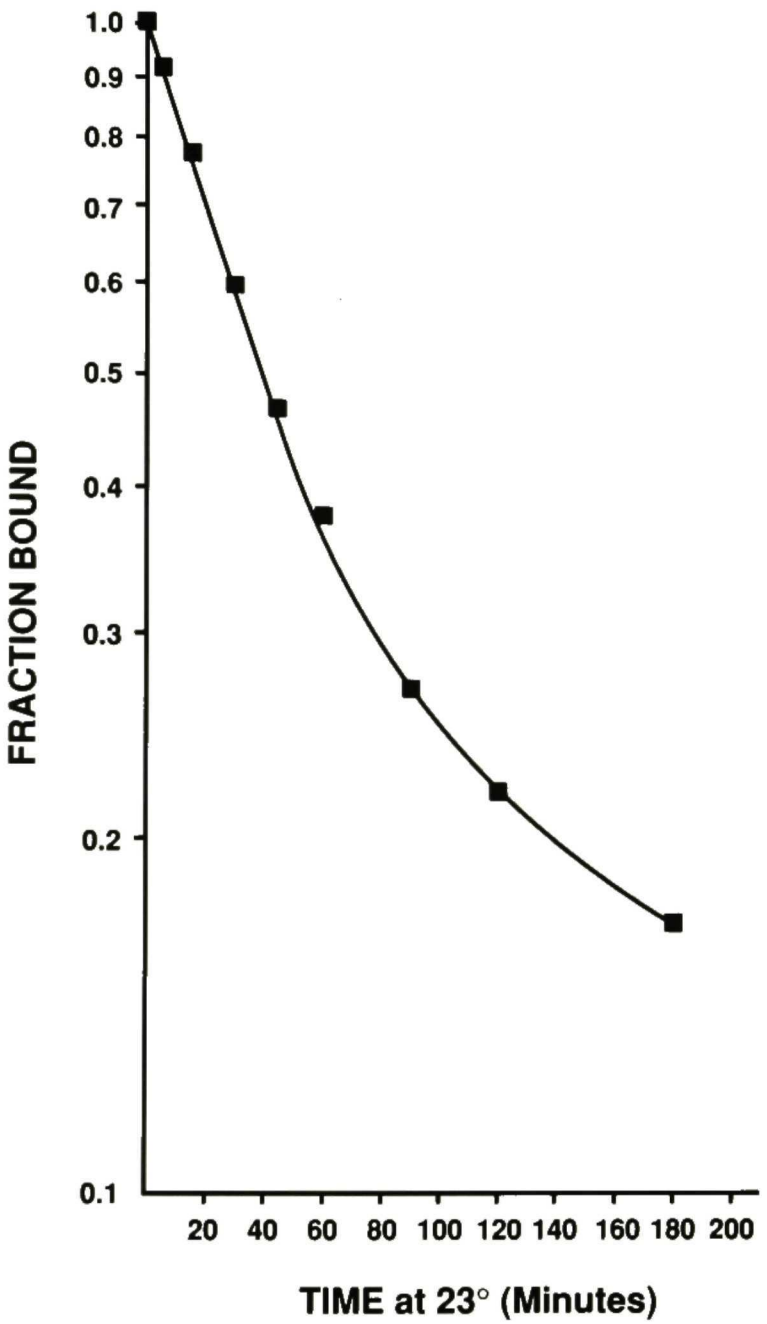
Table 10. Rates of [^{3}H]Dexamethasone Dissociation (k_{off}) for 6TG1.1 and ICR27 at 4°C (with Sodium Molybdate)[a]

Cell line	k_{off} (hour^{-1})		Half life (hour)	
	k_1	k_2	t_1	t_2
6TG1.1	0.028	0.18	25	4
ICR27		0.085		8

[a]The above table summarizes the values obtained from Figure 17. The values are the average of 3 experiments. The k_{off}s of the components in 6TG1.1 were determined as described in Methods.

Figure 18. Steroid dissociation curve for 3R7 at 23°C in the presence of molybdate

Cell extracts of 3R7 were prepared in Buffer 4 containing 20 mM sodium molybdate as described in the Methods Section. Cell extracts were incubated with 5 x 10^{-8} M [^{3}H]dex in the absence or presence of 1 x 10^{-5}M [^{1}H]dex for 2 hours at 23°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. Samples were moved to 23°C and aliquots were taken after various times of incubation at 23°C and analyzed for steroid binding activity. Unbound steroid was removed by the addition of dextran-coated charcoal.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FRACTION BOUND
20 40 60 80 100 120 140 160 180 200
TIME at 23° (Minutes)

The above results are entirely consistent with the original proposition that the GR genes in CEM-C7 are allelic. To test this hypothesis further, the rates of steroid dissociation of 6TG1.1 and ICR27 were compared to the rate of steroid dissociation from receptors in the putatively homozygous human B cell line, IM-9 (Figure 19). In contrast to the rapid off rate seen from ICR27, and the curvilinear off rate seen from 6TG1.1, the rate of steroid dissociation from IM-9 was linear with a relatively slow k_{off} of 0.0066/min. This off rate was nearly identical to the "slow" component seen for 6TG1.1. In fact, when a theoretical dissociation curve was constructed in which it was assumed that the protein encoded by GR^{+} and GR^{*} were equally represented in 6TG1.1 cells, this curve was indistinguishable from the actual experimental data obtained for 6TG1.1. Comparable results were obtained when the dissociation rates were measured at 4°C (Figure 20). Thus, it appears that two allelic forms of the GR gene in 6TG1.1 are indeed expressed.

Section III. Cytotoxicity and Mutagenicity of Adriamycin, Bleomycin and Chlorambucil in CEM-C7 Cells

Glucocorticoids have a cytolytic effect on lymphoid cells of T cell origin (Baxter et al., 1971). This property has led to the use of this class of steroids in the treatment of various leukemias and lymphomas (Claman, 1972; Goldin et al., 1971). Glucocorticoids, by themselves, are effective in inducing remission, but these remissions are short and relapses invariably occur (Viette et al., 1965; Wolff et al., 1967). Further steroid treatment is often ineffective in inducing additional remissions, indicating that resistance to single agent

Figure 19. Steroid Dissociation Curves for IM-9, 6TG1.1, and ICR27, at 23°C in the presence of sodium molybdate.

Cells extracts of 6TG1.1, ICR27, and IM-9 were prepared in Buffer 4 containing 20 mM sodium molybdate as described in Methods. Extracts were incubated with 5 x 10^{-8}M [^{3}H] dex in the absence or presence of 1 x 10^{-5}M [^{1}H]dex for 2 hours at 4°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. The samples were incubated at 23°C and aliquots (200 μl) were taken after various periods of times and assayed for steroid binding activity. Unbound steroid was removed by the addition of dextran coated charcoal. A theoretical curve for 6TG1.1 (- - - -) was constructed using the values of k_{off} obtained from IM-9 and ICR27 and the equation $Y_t/Y_o = (e^{-k1t} + e^{-k2t})/ 2$ where Y_t = binding at time t, Y_o = binding at time 0, t = time at 23°C, $k_1 = k_{off}$ for IM9 at 23°C and $k_2 = k_{off}$ for ICR27 at 23°C. The equation assumes that the components contained in 6TG1.1 are present in equal amounts. The curves for ICR27 and the experimental values for 6TG1.1 are the same as illustrated in Figure 15 and are used for comparison.

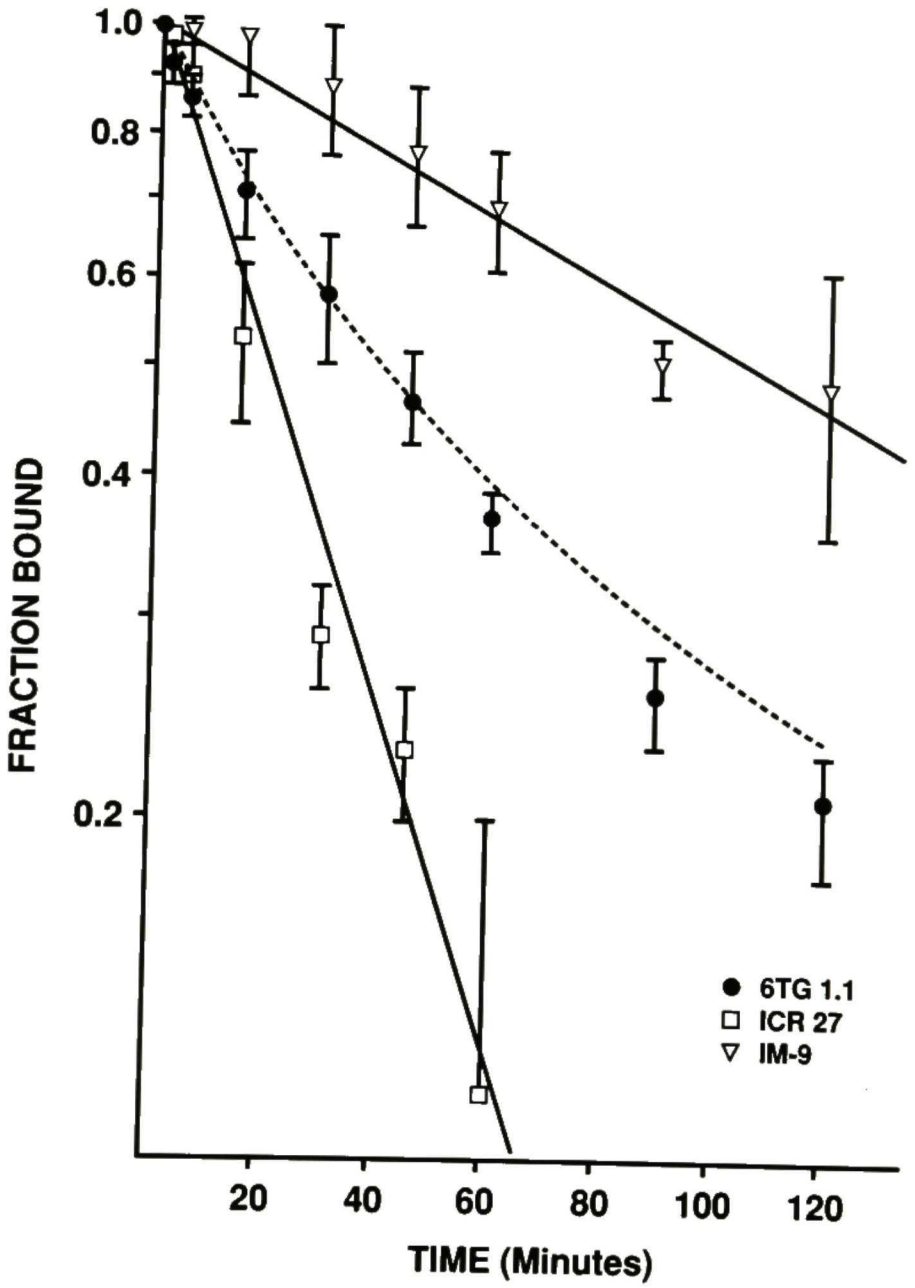
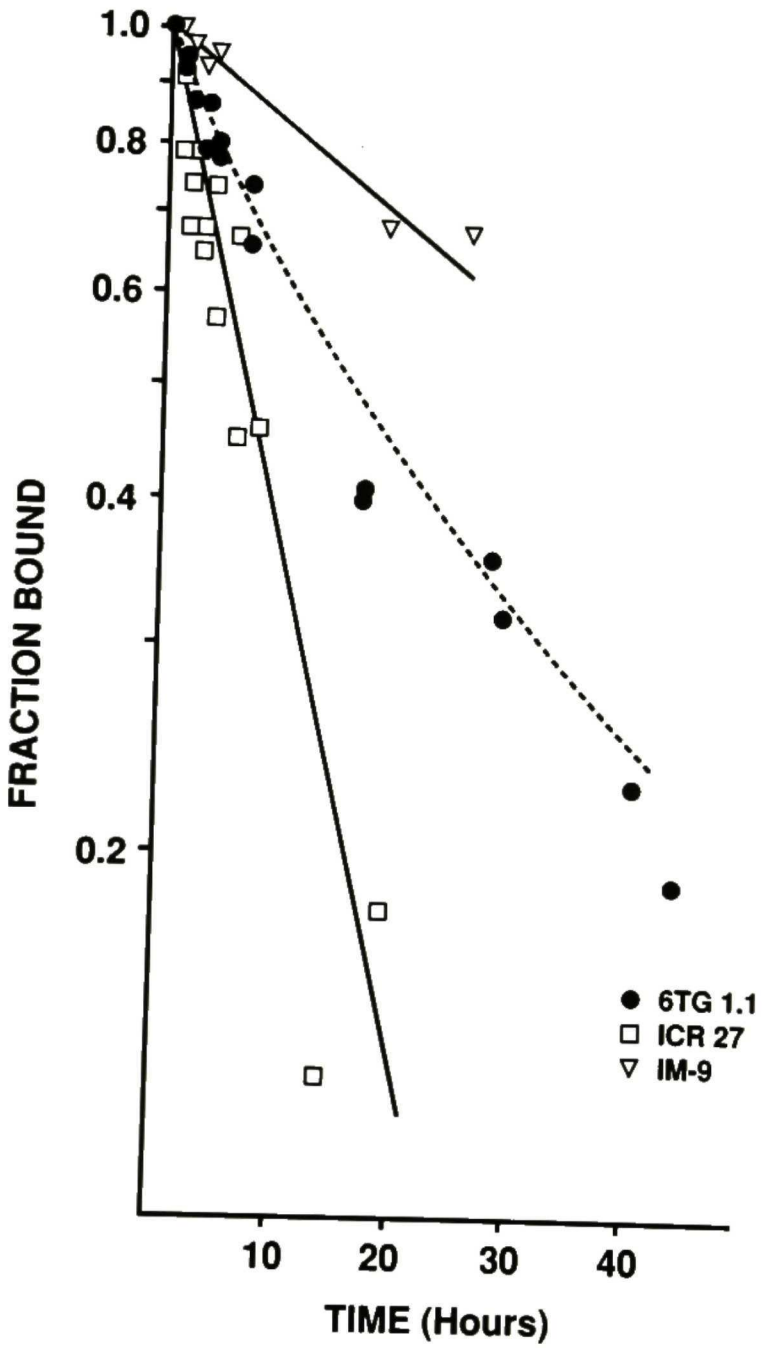

1.0
0.8
0.6
0.4
0.2
FRACTION BOUND
20
40
60
80
100
120
TIME (Minutes)
6TG 1.1
ICR 27
IM-9

Figure 20. Steroid Dissociation Curves for IM-9, 6TG1.1, and ICR27, at 4°C in the presence of sodium molybdate.

Cells extracts of 6TG1.1, ICR27, and IM-9 were prepared in Buffer 4 containing 20 mM sodium molybdate as described in Methods. Extracts were incubated with 5 x 10^{-8}M [^{3}H] dex in the absence or presence of 1 x 10^{-5}M [^{1}H]dex for 2 hours at 4°C. After labeling, additional [^{1}H]dex was added to all samples to prevent rebinding of [^{3}H]dex. The samples remained at 4°C and aliquots (200 μl) were taken after various periods of times and assayed for steroid binding activity. Unbound steroid was removed by the addition of dextran coated charcoal. A theoretical curve for 6TG1.1 (- - - -) was constructed using the values of k_{off} obtained from IM-9 and ICR27 and the equation $Y_t/Y_o = (e^{-k1t} + e^{-k2t})/ 2$ where Y_t = binding at time t, Y_o = binding at time 0, t = time at 23°C, $k_1 = k_{off}$ for IM9 at 4°C and $k_2 = k_{off}$ for ICR27 at 4°C. The equation assumes that the components contained in 6TG1.1 are present in equal amounts. The curves for ICR27 and the experimental values for 6TG1.1 are the same as illustrated in Figure 17 and are used for comparison.


1.0
0.8
0.6
0.4
0.2
FRACTION BOUND
10
20
30
40
TIME (Hours)
6TG 1.1
ICR 27
IM-9

glucocorticoid therapy has developed. Modern therapies of these diseases no longer employ single agent glucocorticoid therapy, but rely on combination therapies which often include glucocorticoids. However, many of the drugs used in these combination protocols are known or suspected mutagenic agents. Thus, it is possible that the drugs used could induce mutations leading to steroid resistance.

The steroid-sensitive human leukemic T cell line has been used as a model system to examine drug-induced steroid resistance. The properties of this cell line: human origin (Foley et al., 1965); near normal karyotype (Moore et al., 1985); significant amounts of GR (Norman and Thompson, 1977; Harmon et al., 1979); growth inhibition and glutamine synthetase induction upon steroid treatment (Norman and Thompson, 1977; Harmon and Thompson, 1982), provide a means to select and confirm the existence of steroid-resistant clones resulting from drug-induced mutations. However, CEM-C7 cells spontaneously mutate to steroid resistance at a rate of approximately 1 x 10^{-5}/cell/generation (Harmon and Thompson, 1981). Thus, the background of spontaneously arising steroid-resistant cells increases with continued growth in culture. To minimize the background frequency of steroid-resistant cells and maximize the chance of identifying drug-induced mutants, a fresh subclone (F5) was isolated from 6TG1.1 by limiting dilution and characterized for karyotype, receptor concentration, equilibrium dissociation constant, growth inhibition and glutamine synthetase induction in the presence of dexamethasone. Subclone F5 was found to be similar in all respects to the dex^s parental cell line (Table 11).

The ability of three drugs: adriamycin, bleomycin, and chlorambucil, to induce mutations which render cells resistant to the

Table 11. Characteristics of Clone F5

1. **15,000 steroid binding sites/cell**
2. **$K_d = 1.5 \times 10^{-8}$ M**
3. **Sensitive to the cytolytic actions of glucocorticoids.**
4. **Induction of glutamine synthetase activity by glucocorticoids.**
5. **Pseudodiploid. (Modal chromosome number = 45.)**

cytolytic effects of glucocorticoids was examined. Initial experiments examined the cytotoxicity of each drug on dex^s cells in order to determine the concentration of drug to use for mutagenesis. Cells in logarithmic phase were treated with various concentrations of adriamycin, bleomycin, or chlorambucil for 24 hours. After drug treatment, cells were washed free of drug, grown in the absence of drug for an additional 24 hours and plated in semi-solid agarose. Colony formation in the semisolid agarose was compared to untreated controls and cell viability was expressed as relative plating efficiency. Dose response curves were obtained for each drug by plotting drug concentration versus the relative plating efficiency. The dose response curve for adriamycin showed a steep loss of viability followed by a plateau (Figure 21). From this curve concentrations between 0.25 μM and 2.54 μM were chosen for the mutation induction experiments. The dose response curve for bleomycin was curvilinear (Figure 22). Therefore, two concentrations were chosen for the mutation induction experiments, one corresponding to a 50% cell kill (2 μg/ml) and the other corresponding to a 99% cell kill (70 μg/ml). The dose response curve for chlorambucil displayed a simple log dose relationship (Figure 23), and a concentration of 5 μM, corresponding to a 90% kill, was chosen for the mutation induction experiments.

The ability of each drug to induce mutations rendering cells resistant to the cytolytic actions of glucocorticoids was determined by the ability of the drug-treated cells to form colonies in semisolid agarose in the presence of steroid. Dex^s cells were treated with the concentration of drug determined from the dose response curves for 24 hours, washed free of drug and allowed to grow for an additional 24

Figure 21. Dose Response Curve for Adriamycin.

6TG1.1 cells in logarithmic growth were incubated with various concentrations of adriamycin for 24 hours. Cells were washed free of drug and grown in drug-free media for an additional 24 hours. Cell viability after drug treatment was determined by colony formation in semi-solid agarose as described in Methods. Points represent the mean and SEM for 3 independently treated cultures.

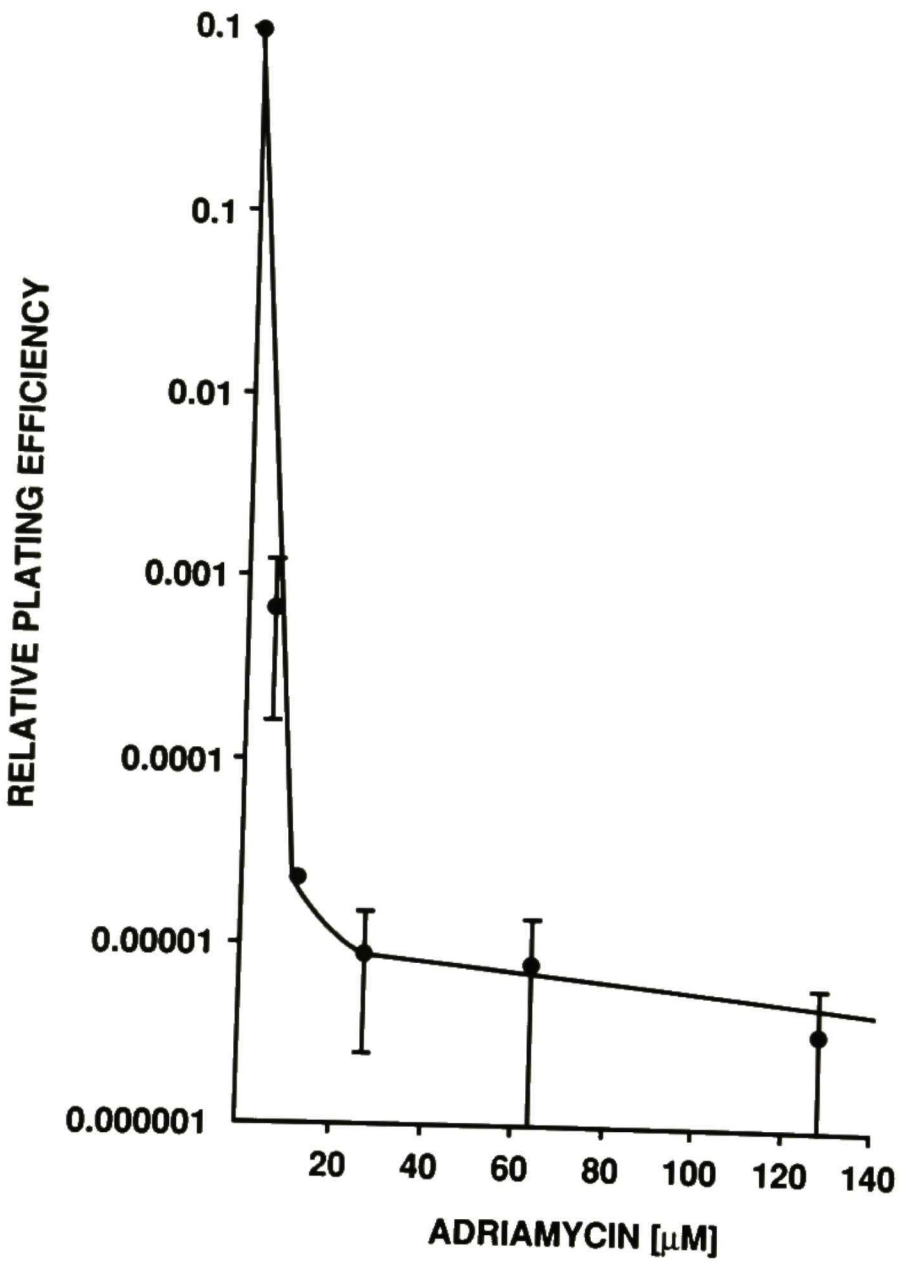

RELATIVE PLATING EFFICIENCY
0.1
0.1
0.01
0.001
0.0001
0.00001
0.000001
20
40
60
80
100
120
140
ADRIAMYCIN [μM]

Figure 22. Dose Response Curve for Bleomycin.

6TG1.1 cells in logarithmic growth were incubated with various concentrations of bleomycin for 24 hours. Cells were washed free of drug and grown in drug-free media for an additional 24 hours. Cell viability after drug treatment was determined by colony formation in semi-solid agarose as described in Methods. Points represent the mean and SEM for 3 independently treated cultures.

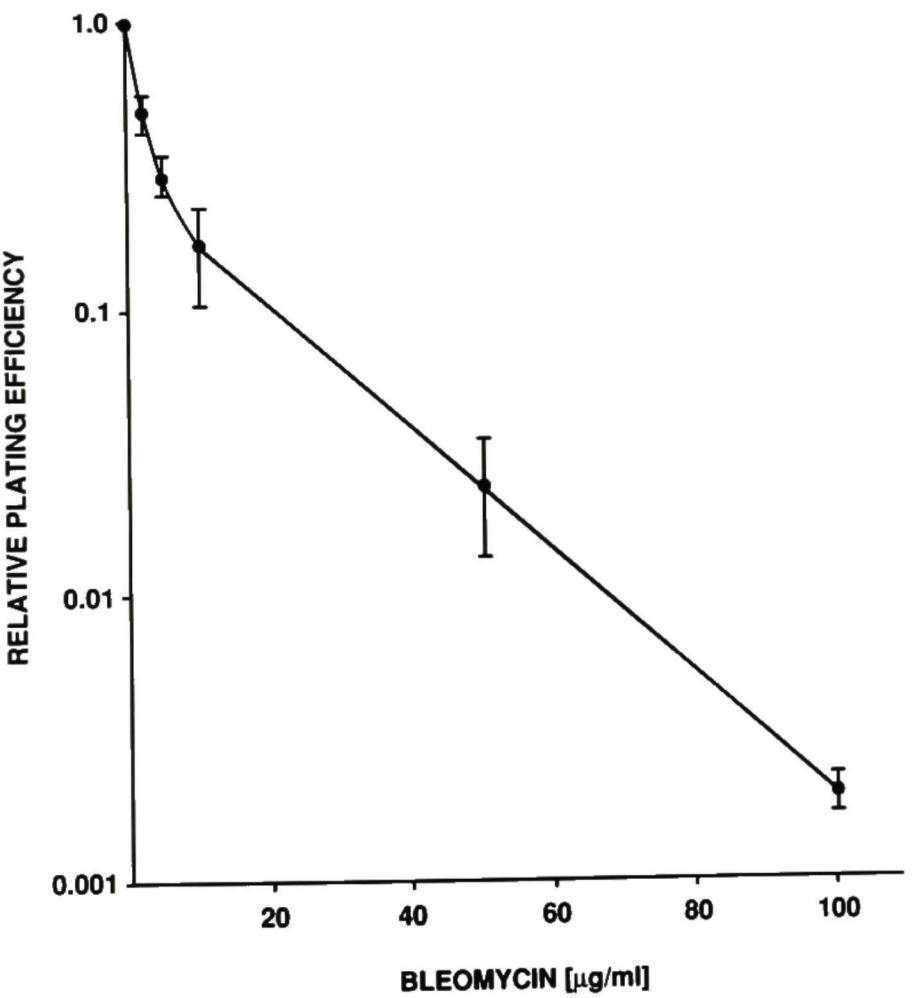
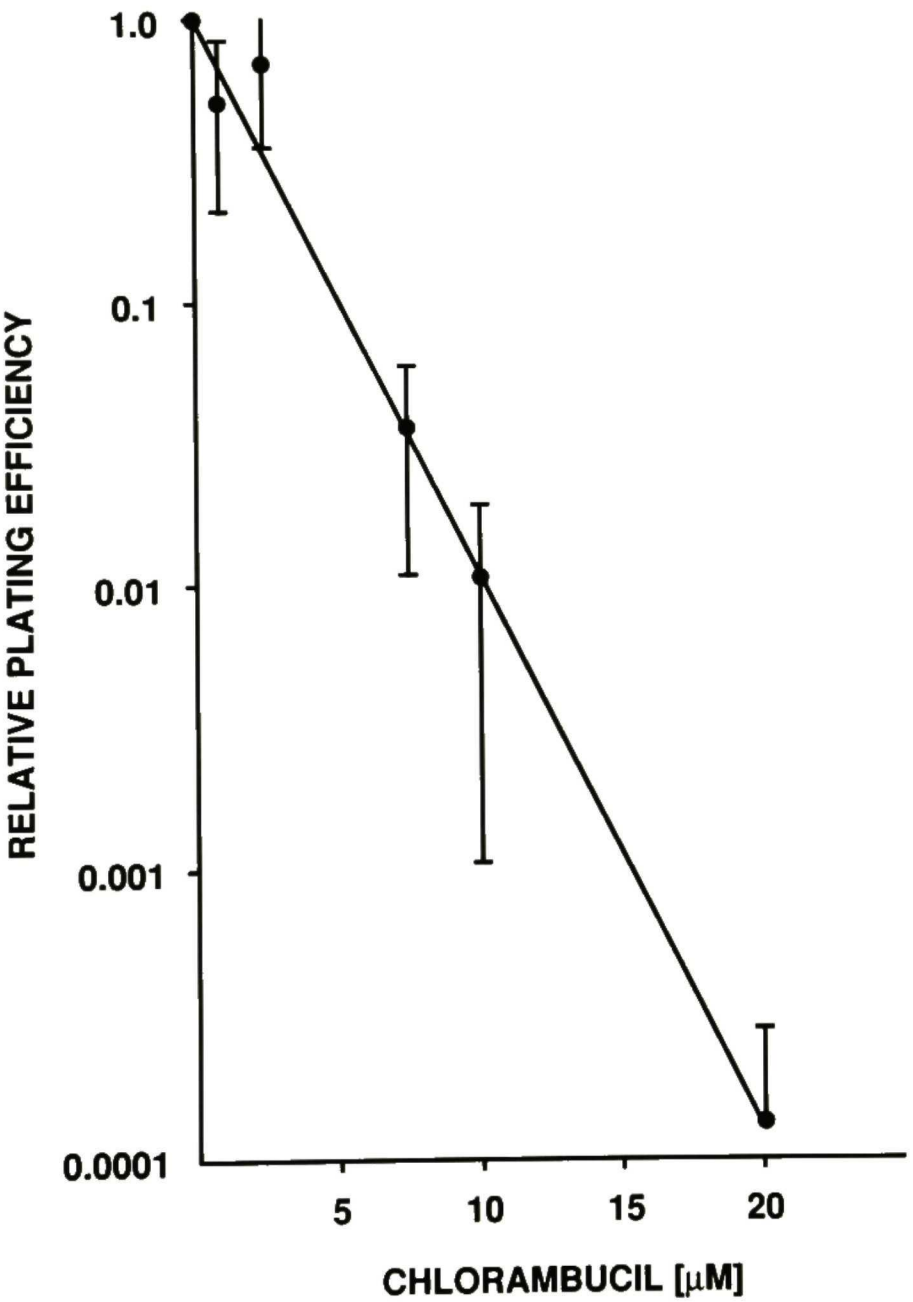

1.0
0.1
0.01
0.001
RELATIVE PLATING EFFICIENCY
20
40
60
80
100
BLEOMYCIN [μg/ml]

Figure 23. Dose Response Curves for Chlorambucil.

6TG1.1 cells in logarithmic growth were incubated with various concentrations of chlorambucil for 24 hours. Cells were washed free of drug and grown in drug-free media for an additional 24 hours. Cell viability after drug treatment was determined by colony formation in semi-solid agarose as described in Methods. Points represent the mean and SEM for 3 independently treated cultures.


1.0
0.1
0.01
0.001
0.0001
RELATIVE PLATING EFFICIENCY
5
10
15
20
CHLORAMBUCIL [μM]

hours to allow expression of the dex^r phenotype (Harmon and Thompson, 1981). Cells were then plated in semisolid agarose in the absence or presence of dexamethasone. Colony formation in the presence of steroid was used to determine steroid resistance. The number of steroid-resistant colonies found after drug treatment was compared to the number of steroid-resistant colonies identified in the absence of drug treatment. A ratio of the (number of steroid-resistant colonies formed $_{\text{drug and steroid treated cultures}}$/number of colonies formed $_{\text{drug treated cultures}}$)/(number of steroid-resistant colonies formed$_{\text{steroid treated cultures}}$/number of colonies formed$_{\text{untreated cultures}}$) was computed and used to define the ability of each drug to induce mutations resulting in steroid resistance above the frequency of spontaneous mutants. Using this criterion, adriamycin (Table 12) and low dose bleomycin (2 μg/ml) (Table 13) did not induce mutations leading to steroid resistance above the spontaneous mutation frequency. On the other hand, high dose bleomycin treatment (70 μg/ml) (Table 13) and chlorambucil treatment (5 μM) (Table 14) were found to be weakly mutagenic, inducing mutations at 2.5 and 5-6 times that of background, respectively. Steroid-resistant clones were isolated from the semi-solid agarose and returned to liquid culture for further characterization.

Section IV. Characterization of the Drug-Induced Steroid-Resistant Cells

Two characteristic GR-mediated responses of CEM-C7 cells to glucocorticoids, the ability of dexamethasone to inhibit growth (Norman and Thompson, 1977) and to induce the activity of the enzyme glutamine synthetase (Harmon and Thompson, 1982), were used to determine if the

Table 12. Induction of Steroid-Resistant Mutants by Adriamycin[a]

Experiment	Dose (μM)	Relative fraction of survivors[b]	Frequency of Dex^r colonies[c]	Fold Induction[d]
1	0.0	1.0	1.35×10^{-4}	-----
	0.254	0.61	2.52×10^{-4}	1.87
2	0.0	1.0	2.55×10^{-4}	-----
	0.254	0.84	2.57×10^{-4}	1.0
	2.54	0.025	1.79×10^{-4}	0.79
3	0.0	1.0	3.23×10^{-4}	-----
	0.384	0.16	4.54×10^{-4}	1.4

[a]F5 cells were treated for 24 hours under the following conditions: no drug or dex, 10^{-6} M dex, the indicated concentration of drug, or the indicated concentration of drug + 10^{-6} M dex as described in Methods. Section. Steroid-resistant cells were seleted by colony formation in dex-containing semi-solid agarose as described in Methods Section.

[b]Calculated as: number of colonies formed$_{\text{drug treated}}$/ number of colonies formed$_{\text{untreated}}$.

[c]Calculated as: (number of dex^r colonies formed$_{\text{drug and steroid treated}}$/ number of colonies formed$_{\text{drug treated}}$)/ (number of dex^r colonies formed$_{\text{steroid treated}}$/ number of colonies untreated).

[d]Calculated as: frequency of dex^r colonies formed$_{\text{drug treated}}$/frequency of dex^r colonies formed$_{\text{untreated}}$.

Table 13. Induction of Steroid-Resistant Mutants by Bleomycin[a]

Experiment	Dose (μg/ml)	Relative fraction of survivors[b]	Frequency of Dex^r colonies[c]	Fold Induction[d]
1	0.0	1.0	3.21×10^{-4}	----
	2.0	1.0	3.33×10^{-4}	1.0
	70.0	0.012	1.18×10^{-3}	3.66
2	0.0	1.0	5.76×10^{-4}	-----
	2.0	0.662	9.81×10^{-4}	1.7
	70.0	0.014	1.46×10^{-3}	2.53
3	0.0	1.0	2.02×10^{-4}	-----
	2.0	0.78	2.36×10^{-4}	1.16
4	0.0	1.0	4.81×10^{-4}	-----
	70.0	0.006	1.17×10^{-3}	2.43

[a]F5 cells were treated for 24 hours under the following conditions: no drug or dex, 10^{-6} M dex, the indicated concentration of drug, or the indicated concentration of drug + 10^{-6} M dex as described in Methods. Steroid-resistant cells were seleted by colony formation in dex-containing semi-solid agarose as described in Methods.

[b]Calculated as: number of colonies formed$_{drug\ treated}$/ number of colonies formed$_{untreated}$.

[c]Calculated as: (number of dex^r colonies formed$_{drug\ and\ steroid\ treated}$/ number of colonies formed$_{drug\ treated}$)/ (number of dex^r colonies formed$_{steroid\ treated}$/ number of colonies untreated).

[d]Calculated as: frequency of dex^r colonies formed$_{drug\ treated}$/frequency of dex^r colonies formed$_{untreated}$.

Table 14. Induction of Steroid-Resistant Mutants by Chlorambucil[a]

Experiment	Dose (μM)	Relative fraction of survivors[b]	Frequency of Dex^r colonies[c]	Fold Induction[d]
1	0.0	1.0	5.49×10^{-5}	-----
	5.0	0.111	3.15×10^{-4}	5.3
2	0.0	1.0	1.94×10^{-4}	-----
	10.0	0.0037	1.19×10^{-3}	6.1

[a]F5 cells were treated for 24 hours under the following conditions: no drug or dex, 10^{-6} M dex, the indicated concentration of drug, or the indicated concentration of drug + 10^{-6} M dex as described in Methods. Steroid-resistant cells were seleted by colony formation in dex-containing semi-solid agarose as described in Methods.

[b]Calculated as: number of colonies formed$_{drug\ treated}$/ number of colonies formed$_{untreated}$.

[c]Calculated as: (number of dex^r colonies formed$_{drug\ and\ steroid\ treated}$/ number of colonies formed$_{drug\ treated}$)/ (number of dex^r colonies formed$_{steroid\ treated}$/ number of colonies untreated).

[d]Calculated as: frequency of dex^r colonies formed$_{drug\ treated}$/frequency of dex^r colonies formed$_{untreated}$.

dex^r phenotypes were the result of a defect in the GR or in some other component in the pathway of steroid response. The ability of these cells to grow in the presence of dexamethasone was examined by measuring the growth in liquid culture of cells cultured in the absence or presence of 1×10^{-6} M dex (Figure 24). Growth of the drug-induced steroid-resistant cells was not affected by the presence of dex (Panels B and C), whereas, growth of the dex^s parental cell line was significantly inhibited (Panel A).

The ability of dex to induce the activity of glutamine synthetase was also examined (Table 15). Little if any increase in enzyme activity was seen in the drug-induced steroid-resistant cells while there was a significant response in the parental cells. Thus, since growth inhibition and glutamine synthetase induction in the presence of dexamethasone are independent events both mediated through the GR (Harmon and Thompson, 1982), the loss of these two unlinked responses suggests the loss of a common element. Based on previous results, it seemed that a defect in the GR was the most likely candidate.

Steroid-resistant cells previously isolated from the CEM-C7 cells have been characterized as having one of two receptor phenotypes: 1) $\underline{r}^-$, characterized by the inability to bind steroid hormone under physiological conditions; and 2) $\underline{act}^1$, characterized by the inability of the receptor to retain ligand after receptor activation. However, after chemical mutagenesis, only the $\underline{r}^-$ phenotype was isolated. Therefore, initial experiments examined the ability of drug-induced steroid-resistant cells to bind steroid hormone. [H^3]Dexamethasone binding in whole cell preparations demonstrated that both bleomycin and

Figure 24. Dexamethasone Effects on cell Growth.

Cells were grown in the absence (●) or presence (○) of 1 x 10^{-6} M dexamethasone. Cell number was determined daily. The growth of clone F5 (A) was inhibited by the presence of dexamethasone, while growth is not affected in CHL A1 (B) or BLM #1 (C).

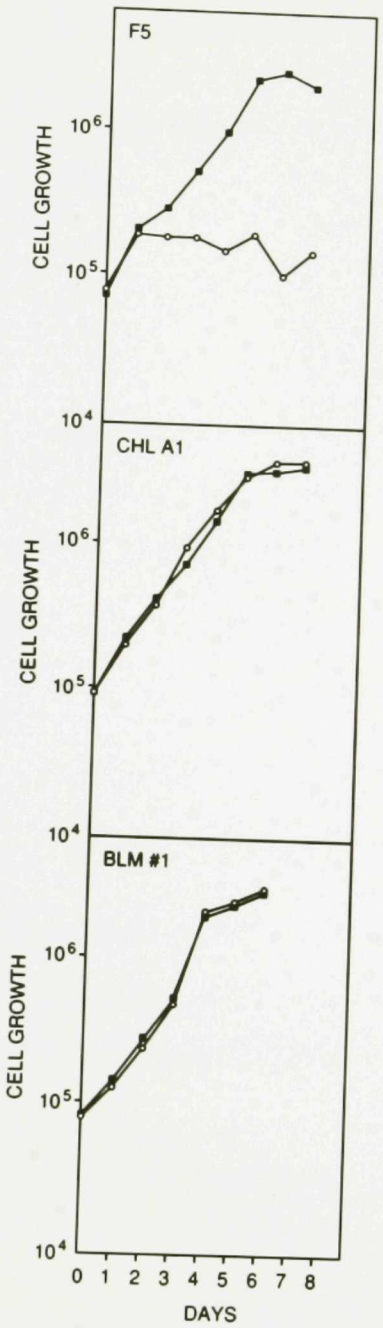

F5
CHL A1
BLM #1
CELL GROWTH
10^6
10^5
10^4
0 1 2 3 4 5 6 7 8
DAYS

Table 15. Glutamine Synthetase Activity in Dexamethasone-Treated Bleomycin- and Chlorambucil-Induced Steroid-Resistant Cells[a]

Clone	Glutamine Synthetase Activity[b] Basal[c]	Induced	Induction[d]
F5 (wild type)	5.2 ± 0.3	17.3 ± 0.7	3.4
Chl A1	4.1 ± 0.1	3.5 ± 0.3	0.8
Chl A2	4.2 ± 0.2	3.8 ± 0.6	0.9
Chl A3	3.6 ± 0.1	3.7 ± 0.3	1.0
Chl A4	3.6 ± 0.2	4.1 ± 0.3	1.1
Chl A5	2.3 ± 0.3	2.7 ± 0.2	1.2
Chl A6	1.5 ± 0.2	2.0 ± 0.1	1.3
Chl #6	4.8 ± 0.5	4.8 ± 0.4	1.0
Chl #13	5.6 ± 0.3	6.6 ± 0.6	1.2
Blm B2	2.2	2.8	1.3
Blm B3	1.6	1.5	0.9
Blm B4	2.7	3.3	1.2
Blm B5	1.3	1.1	0.8
Blm B6	2.1	3.0	1.4
Blm C5	1.7	2.3	1.4
Blm C6	1.9	2.3	1.2
Blm #1	12.7	13.3	1.0

[a]Cells were treated with 10^{-6} M dex or an equal volume of ethanol for 18-24 hours and then assayed for the amount of γ-glutamyl transferase activity as described in Methods Section

[b]The glutamine synthetase activity is expressed as micromoles of glutamine converted to γ-glutamyl-hydroxymate/min/mg protein.

[c]The values for the chlorambucil-induced steroid-resistant cells are the mean ± SEM for 3 experiments. The values for the bleomycin-induced steroid-resistant cells are the values for 1 experiment. Experiments with Blm #1 were separate from the other cells. In these experiments, basal levels were elevated in all cells tested.

[d]Induced/Basal.

chlorambucil-induced dex^r cells had reduced steroid binding activity compared to dex^s parental cells. In addition, a majority of the drug-induced dex^r cells had less than 10% of the steroid binding activity seen in the dex^s parental cells (Figures 25 and 26). By convention, these cells were therefore classified as having an $\underline{r}^-$ phenotype. The steroid binding activity in these drug-induced dex^r cells was also determined in cell extracts. Like the dex^r $\underline{r}^-$ cell line ICR27, drug-induced steroid-resistant cells contained significantly more steroid binding activity when steroid binding was determined in cell extracts (Table 16). However, the amount of steroid binding was still reduced when compared to dex^s parental cells.

To further characterize the GR present in these drug-induced dex^r cells, extracts prepared from [^{3}H]DM-labeled bleomycin and chlorambucil-induced dex^r cells were fractionated by SDS-PAGE and the steroid binding protein visualized by fluorography. The steroid binding protein present in bleomycin (not shown) and chlorambucil-induced (Figure 27) dex^r cells migrated as a 92 kDa band indistinguishable from the GR in dex^s parental cells. This band was eliminated if the labeling reaction was performed in the presence of excess [^{1}H]dex. Inspection of these results indicated that there was somewhat less [^{3}H]DM-labeled material in the drug-induced dex^r cells than in the dex^s parent. However, more [^{3}H]DM binding activity appeared to be present than would be expected on the basis of the whole cells binding results.

Immunochemical analysis of the GR in drug-induced dex^r cells confirmed these results. Cell extracts were fractionated by SDS-PAGE and the immunoreactive GR protein detected by immunoblot using the anti-GR antibody AC40 (Eisen et al., 1988) and ^{125}I protein A (Figure 28).

Figure 25. Whole cell binding Analysis of Bleomycin-Induced Steroid-Resistant Cells.

Whole cells were incubated with 5 x 10^{-8} M [^{3}H]dexamethasone in the absence or presence of excess of [^{1}H]dexamethasone at 37°C for 60 min as described in Methods. All of the bleomycin-induced steroid-resistant cells isolated contain reduced amounts of steroid binding activity. In addition, the majority of these cells contain less than 10% of the steroid binding activity of the parental steroid-sensitive clone F5.

DEXAMETHASONE BINDING ACTIVITY
IN BLEOMYCIN - INDUCED MUTANTS
DEXAMETHASONE BINDING ACTIVITY
(% OF CONTROL)
0
10
20
30
BLM A2
BLM A4
BLM A5
BLM B1
BLM B2
BLM B4
BLM B5
BLM B6
BLM C5
BLM C6
BLM #1
CLONES

Figure 26. Whole cell binding Analysis of Chlorambucil-induced steroid-resistant cells.

Whole cells were incubated with 5 x 10^{-8} M [^{3}H]dexamethasone in the absence or presence of excess of [^{1}H]dexamethasone at 37°C for 60 min as described in Methods. All of the chlorambucil-induced steroid-resistant cells isolated contain reduced amounts of steroid binding activity. In addition, the majority of these cells contain less than 10% of the steroid binding activity of the parental steroid-sensitive clone F5.

DEXAMETHASONE BINDING ACTIVITY
IN CHLORMABUCIL - INDUCED MUTANTS
DEXAMETHASONE BINDING ACTIVITY
(% OF CONTROL)
50
40
30
20
10
0
CHL A1
CHL A2
CHL A3
CHL A4
CHL A5
CHL A6
CHL #6
CHL #13
CLONES

Table 16. Binding of[^{3}H]Dexamethsone in Cell Extracts of Bleomycin- and Chlorambucil-Induced Steroid-Resistant Cells[a]

Cell line	Binding Activity (fmol/mg protein)	% Control
F5	116	1.00
Chl A1	68	0.59
Chl #13	76	0.66
Blm #1	53	0.46
Blm B4	62	0.53

[a]Cell extracts were prepared in Buffer 4 containing 20 mM sodium molybdate as described in Methods Section. Extracts (200 µl) were incubated with 5×10^{-8} M [^{3}H] dex for 2 h at 4^{o}C. Specific [^{3}H]dex binding was determined as described in Methods. The values represent the average of two experiments.

Figure 27. [^{3}H]Dexamethasone Mesylate Labeling of the Glucocorticoid Receptor in Chlorambucil-Induced Steroid-Resistant Cells.
Cells were incubated with the covalent affinity ligand [^{3}H]dexamethasone in the absence (U) or presence (C) of excess [^{1}H]triamcinolone acetonide as described in Methods. GR was visualized by fluorography. A lane of [^{14}C] labeled marker proteins is indicated on the left.

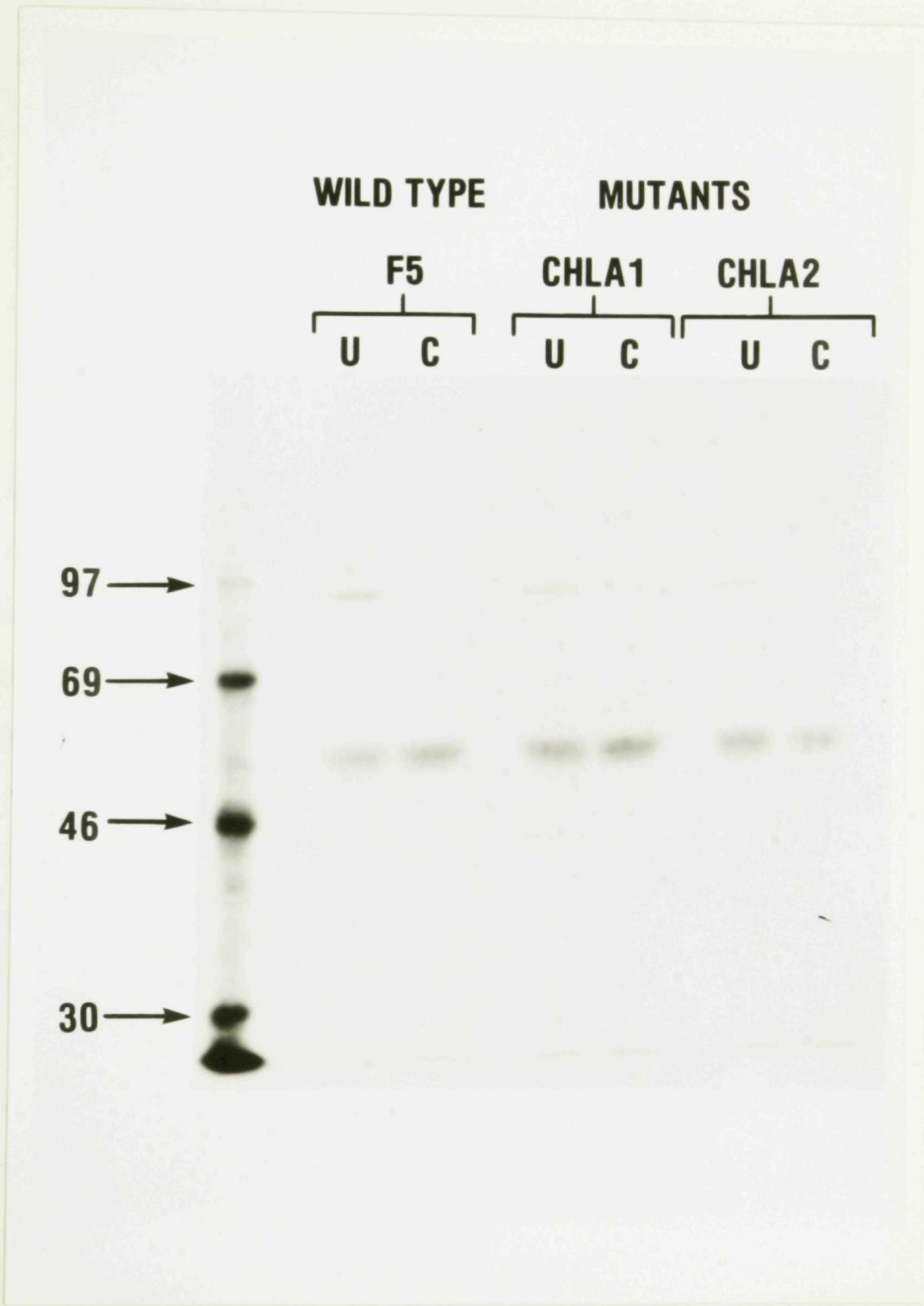
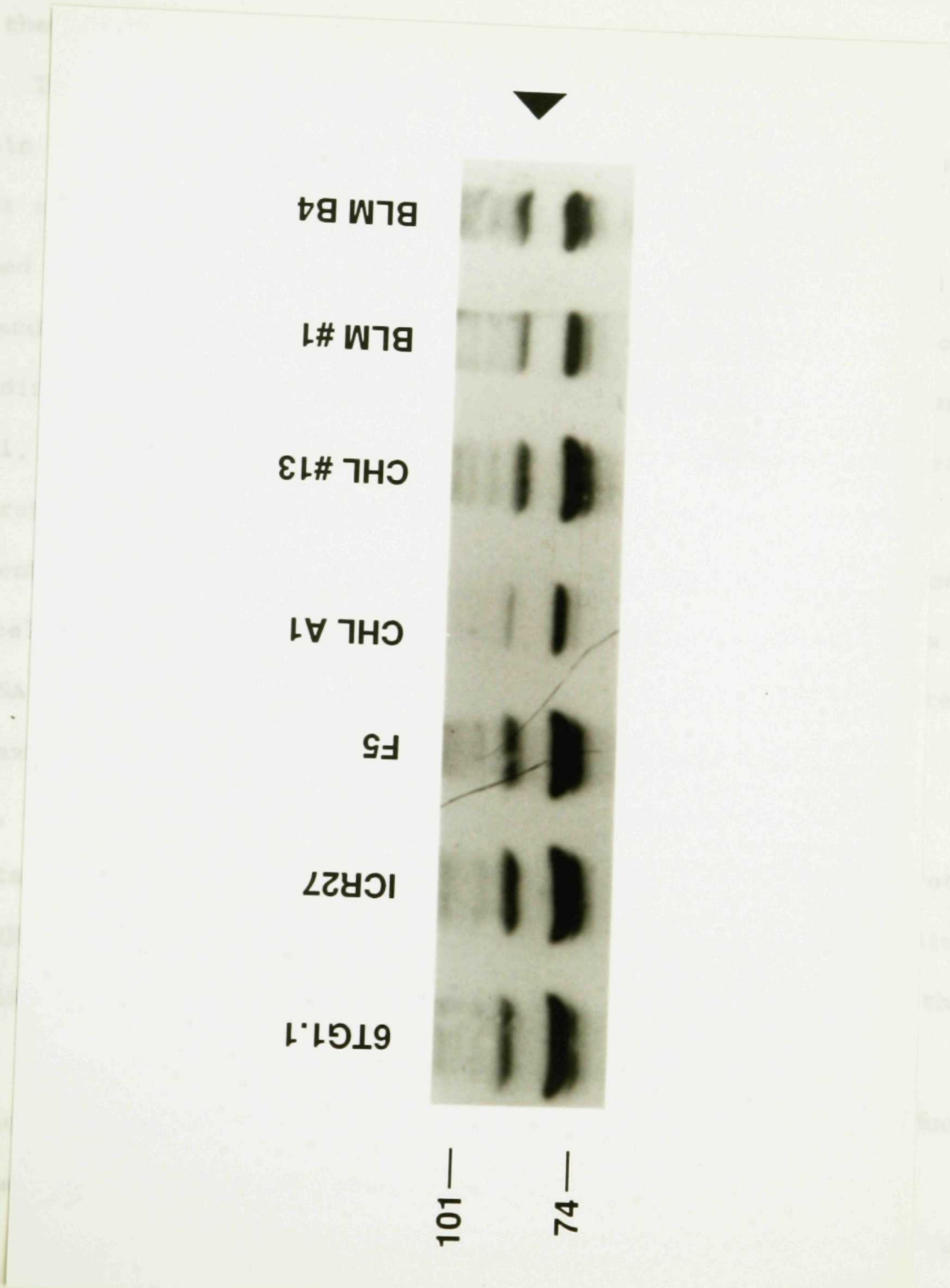

WILD TYPE
MUTANTS
F5
CHLA1
CHLA2
U
C
U
C
U
C
97
69
46
30

Figure 28. Immunoblot analysis of Bleomycin- and Chlorambucil-induced steroid-resistant cells.

Cells extracts were prepared as described in Methods. The extracts were fractionated by SDS-PAGE and transferred to nitrocellulose as described in Methods. The immunoreactive GR protein was visualized by the anti-GR antibody AC40 and ^{125}I protein A.


BLM B4
BLM #1
CHL #13
CHL A1
F5
ICR27
6TG1.1
101
74

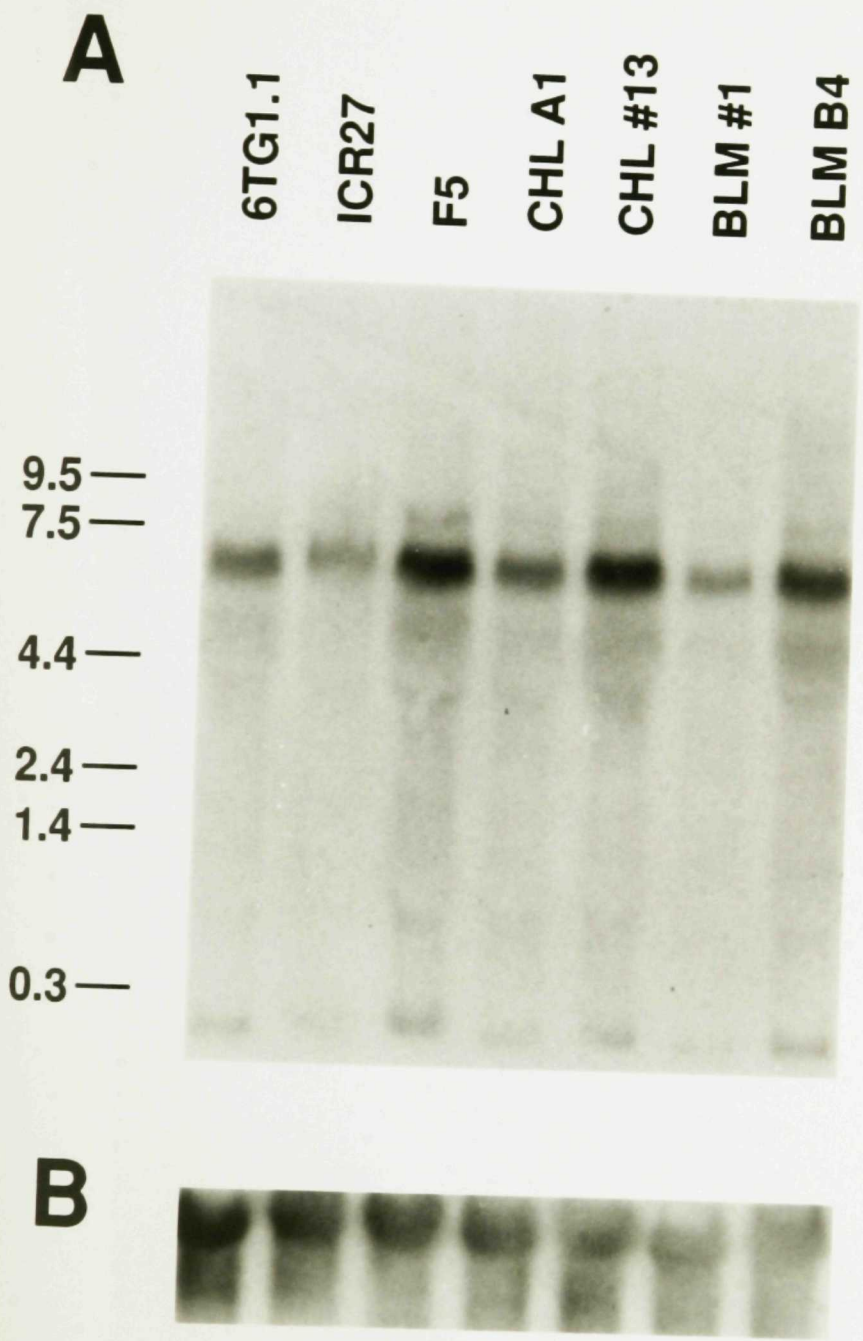
The immunoreactive protein in these cells was indistinguishable in size from the dex^s parental cells, migrating at 92 kDa, thus confirming the identity of the [^{3}H]DM labeled material as the GR. In general, it appeared that the dex^r mutants expressed less immunoreactive GR protein than their dex^s parents.

To determine if the apparent reduction in the amount of GR protein was paralleled by a reduction in the amount of GR mRNA produced and/or a change in mRNA size, RNA isolated from dex^s and the drug-induced dex^r cells was analyzed by Northern blot analysis. RNA from dex^s and dex^r cells grown in serum stripped medium was isolated by acid guanidinium-thiocyanate phenol-chloroform extraction (Chromczynski and Sacchi, 1987), fractionated on a 1% agarose gel, transferred to Nytran, and probed with a 402 bp GR-specific probe isolated from phGR2.9 (Hollenberg et al., 1985). The GR mRNA isolated from the drug-induced dex^r cells migrated as a prominent 7 kb band, indistinguishable from the GR mRNA present in the dex^s cells (Figure 29). As previously reported for dex^r clones isolated after ICR-191 mutagenesis (Harmon et al., 1989), drug-induced dex^r clones expressed significant, but variable amounts of GR mRNA (Figure 29), making it difficult to correlate protein and mRNA levels. However, taken together, the analysis of GR protein and mRNA in dex^r clones is consistant with our previous hypothesis that the $\underline{r}^-$ phenotype is the result of a mutation in the GR$^+$ allele. In addition, it suggests that the GR immunoreactivity seen in drug-induced dex^r cells is probably the product of the GR* allele.

In order to detect potential deletions or rearrangements in the GR gene(s) in drug-induced dex^r cells, the genomic organization of the human GR gene in dex^s and dex^r cells was examined by Southern blot

Figure 29. Northern Analysis of bleomycin and chlorambucil-induced Steroid Resistant cells.

Total RNA was isolated from cells by acid guanidinium thiocyanate-phenol-chloroform extraction (Chromczynski and Sacci, 1987) as described in Methods. Total RNA (5 ug) was denatured with glyoxyl and fractionated on a 1% agarose gel in 0.01 M sodium phosphate as described in Methods. A. Blots were probed with a 402 bp Eco RI fragment of phGR2.9 (Hollenberg et al., 1985). B. Blots were stripped and re-probed with the 1.1 kb Eco RI/Bam HI fragment of p5B (Bowman et al., 1981) specific for 18S rRNA.


A
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
9.5
7.5
4.4
2.4
1.4
0.3
B

analysis. Genomic DNA isolated from dex^s and dex^r cells was digested to completion with six restriction endonucleases. Three of these enzymes (Bam HI, Bcl I, and Xba I) do not cleave within the coding sequence of the hGR. The others (Eco RI, Hind III, and Pst I) cleave the human GR within the coding region and are divided into two groups based on their cleavage 5' or 3' from the Cla I site. Two probes, a 1.6 kbp Pst I/Cla I fragment and the 1.3 kbp Cla I/Xba I fragment, were isolated from pGR107, which contains the complete cDNA for hGR alpha (Hollenberg et al., 1985). The 1.6 kbp Pst I/Cla I fragment contains the sequence encoding part of the 5' untranslated region, the amino terminal domain, the first zinc finger and the proximal portion of the second zinc finger. The 1.3 kbp Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain, and a portion of the 3' untranslated region (Figure 30). Restriction patterns obtained after digestion with Bam HI (Figure 31), Eco RI (Figure 32), Hind III (Figure 33), Pst I (Figure 34), and Xba I (Figure 35) showed no difference in the genomic organization of the GR genes in dex^s and in the bleomycin- and chlorambucil-induced dex^r cells. These results are consistent with previous studies which showed no differences in the genomic organization of the hGR gene in dex^s r^+ and dex^r r^- CEM cells as well as between CEM and normal human genomic DNA (Harmon et al., 1989).

When the Pst I /Cla I fragment was used to probe Bcl I digests however, a distinct difference was seen in two of five dex^r clones. In each of these clones (ICR27 and Blm #1) a 4.4 kbp band was completely absent (Figure 36). A Bcl I restriction fragment length polymorphism has previously been reported for the hGR gene, yielding hybridizing fragments of either 2.3 or 4.5 kbp (Murray et al., 1987). Since Bcl I

Figure 30. Organization of the human GR gene

A. Schematic representation of the exons encoding the human GR. Demarcation for exon 1 and exon 2 are based on the work by Zong et al. (1990). The intron-exon boundaries for the other exons are unknown at this time. However, exons 3 and 4 encode for the two zinc finger structures located in the DNA binding domain (Enrico et al., 1990). B. Schematic representation of the coding sequence for the human GR obtained from pGR107 (Hollenberg et al., 1985) illustrating the indicated restriction sites. Thin lines represent vector sequence, thick lines represent untranslated regions, and the open bar represents the coding sequence. C. Location of the two probes used in Southern blot analysis. The 1.6 kb Pst I/Cla I fragment includes a portion of the 5' untranslated region, the amino terminal domain, first zinc finger and the proximal portion of the second zinc finger. This would include sequence encoded by exons 1-4. The 1.3 Cla I/Xba I fragment contains the distal end of the second zinc finger, the steroid binding domain, and a part of the 3' untranslated region encoded by exons 4-10. D. The location of the 402 bp fragment of phGR 2.9.

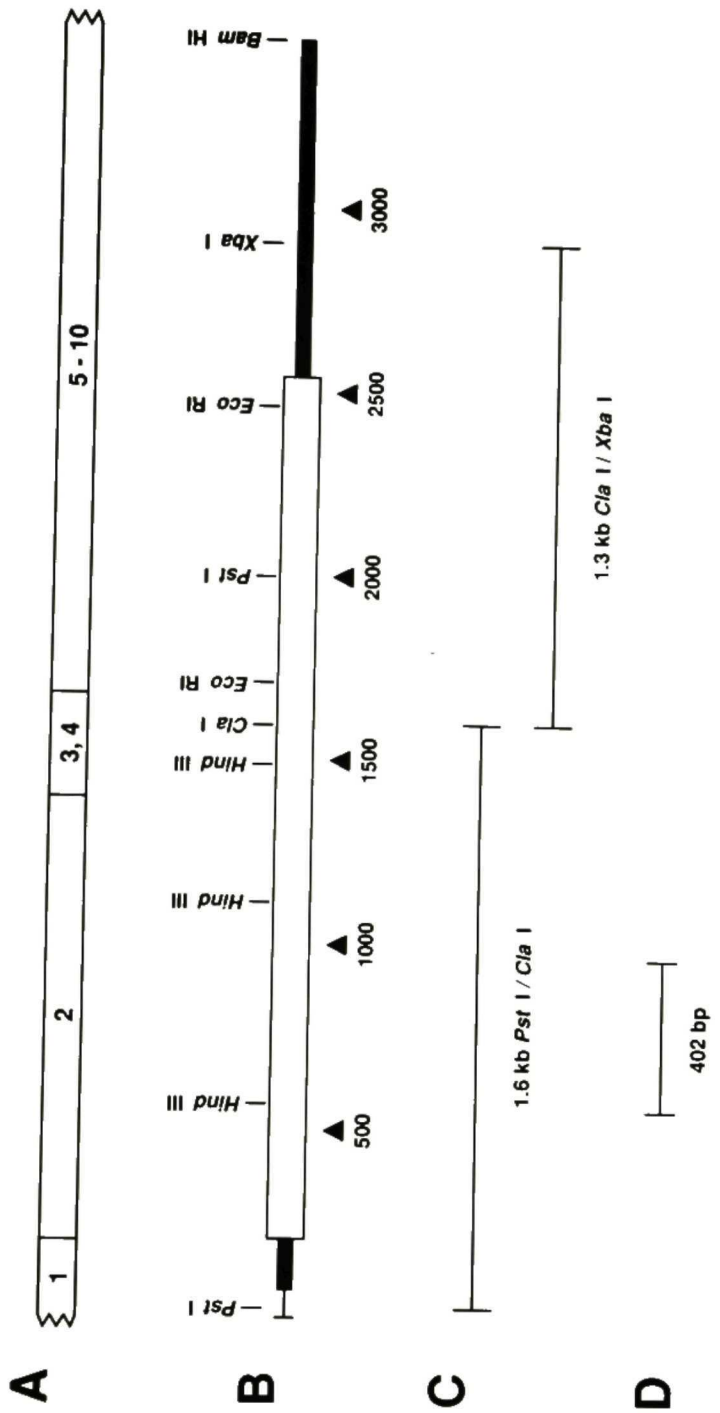

A
1
2
3, 4
5 - 10
B
Pst I
Hind III
Hind III
Hind III
Cla I
Eco RI
Pst I
Eco RI
Xba I
Bam HI
500
1000
1500
2000
2500
3000
C
1.6 kb Pst I / Cla I
1.3 kb Cla I / Xba I
D
402 bp

Figure 31. Southern Analysis of Bam HI digests.

Genomic DNA was digested to completion with Bam HI, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.

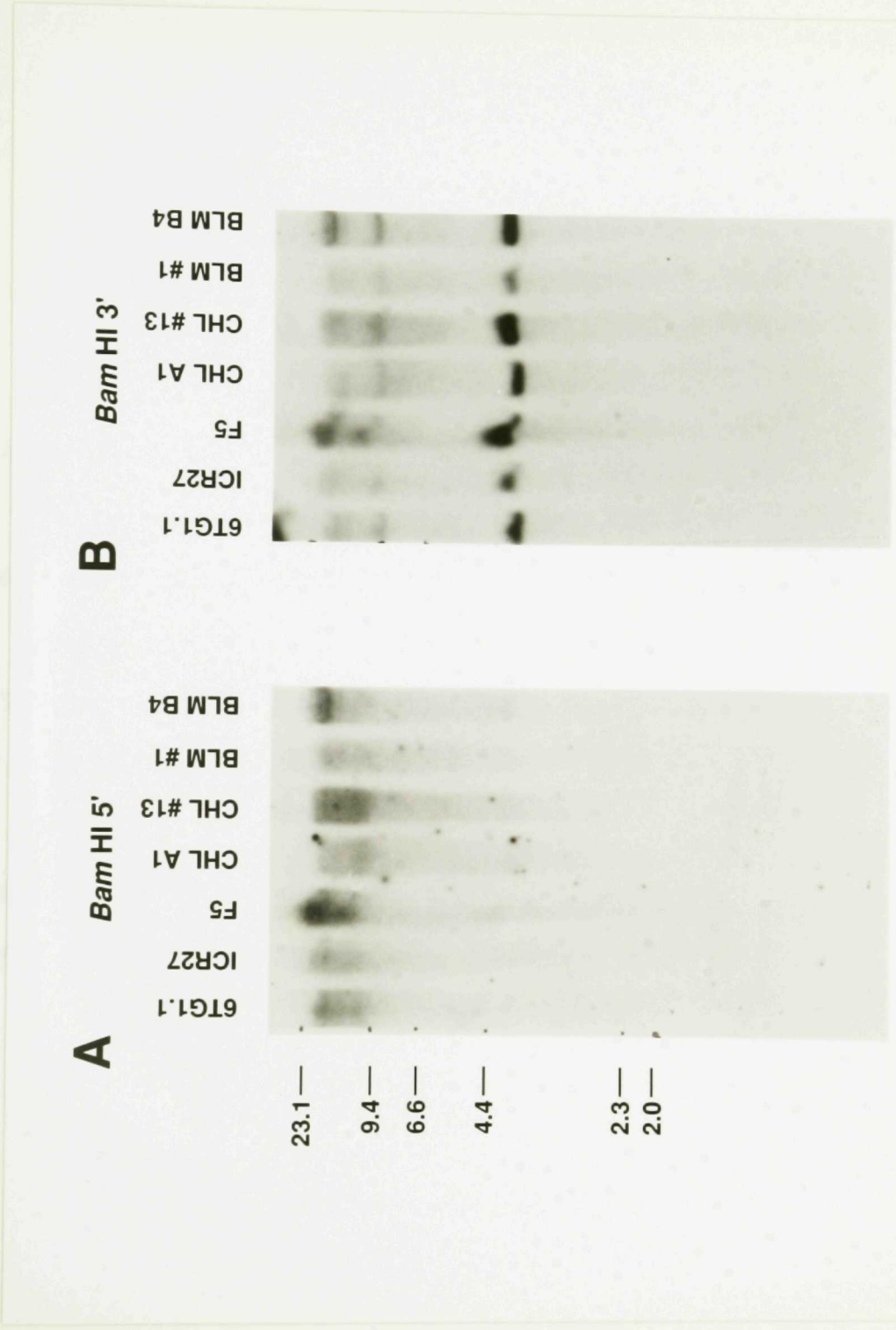

A
Bam HI 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Bam HI 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

Figure 32. Southern Analysis of Eco RI digests.

Genomic DNA was digested to completion with Eco RI, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.

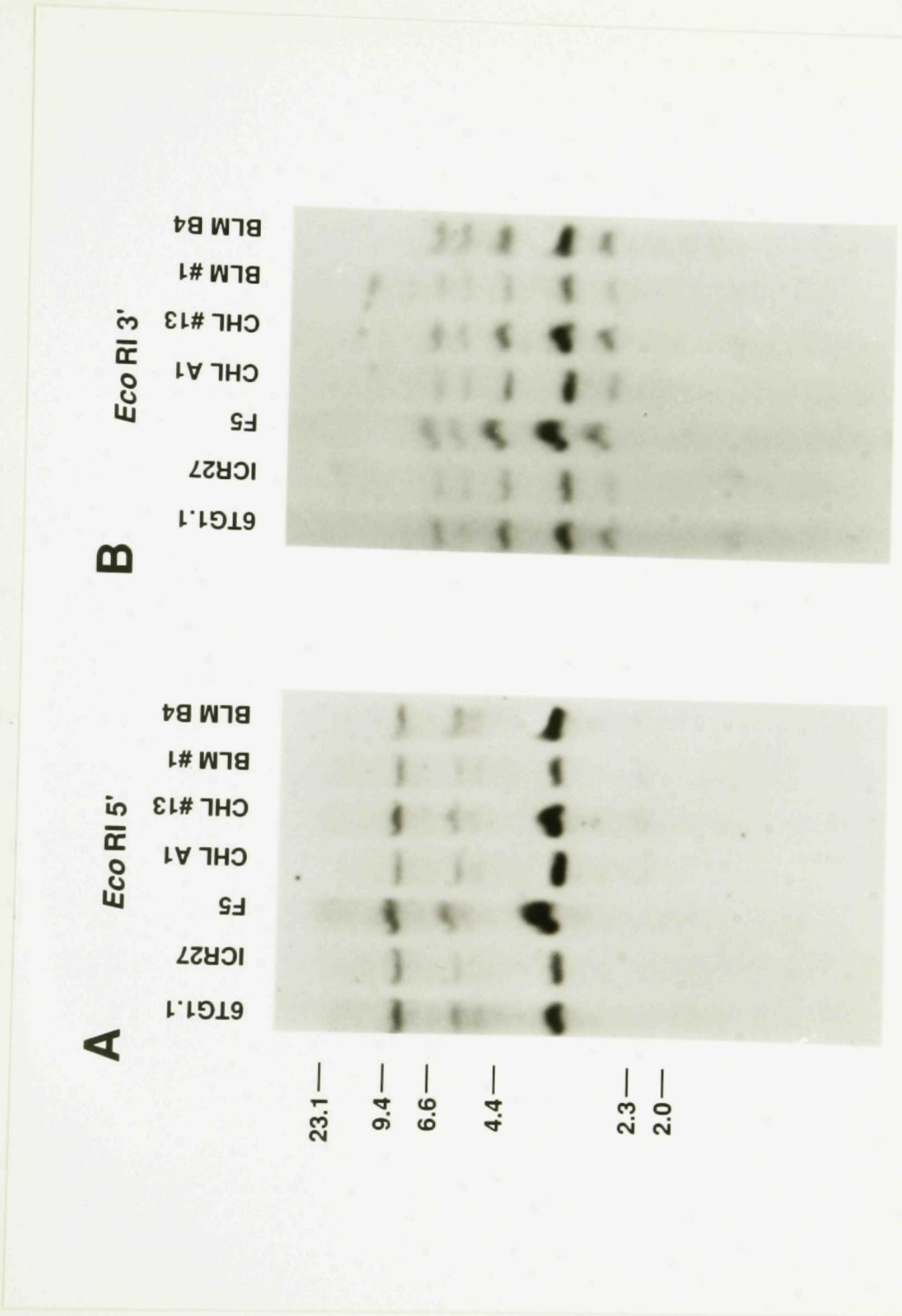

A
Eco RI 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Eco RI 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

Figure 33. Southern Analysis of Hind III digests.

Genomic DNA was digested to completion with Hind III, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.

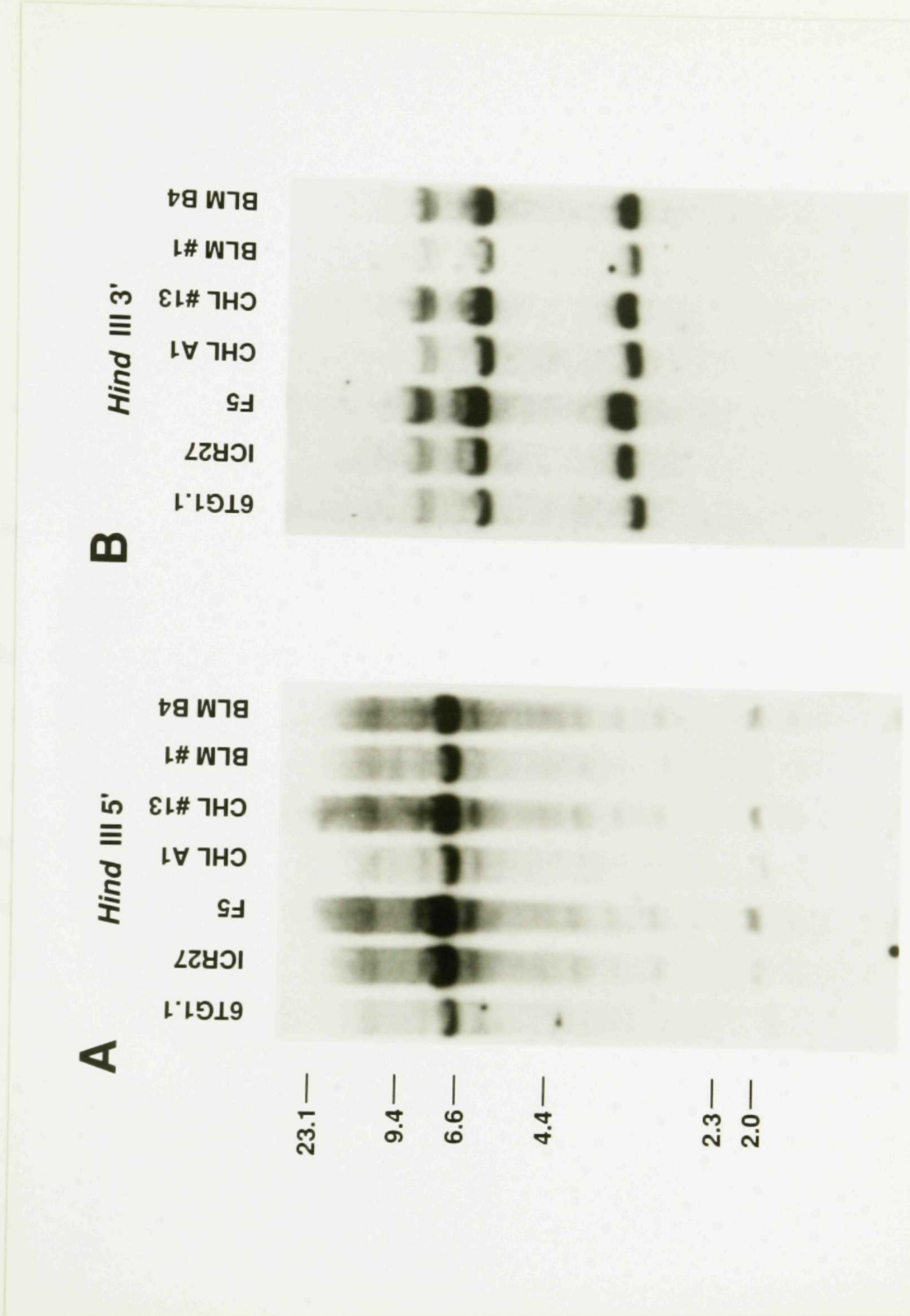

A
Hind III 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Hind III 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

Figure 34. Southern Analysis of Pst I digests.

Genomic DNA was digested to completion with Pst I, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.

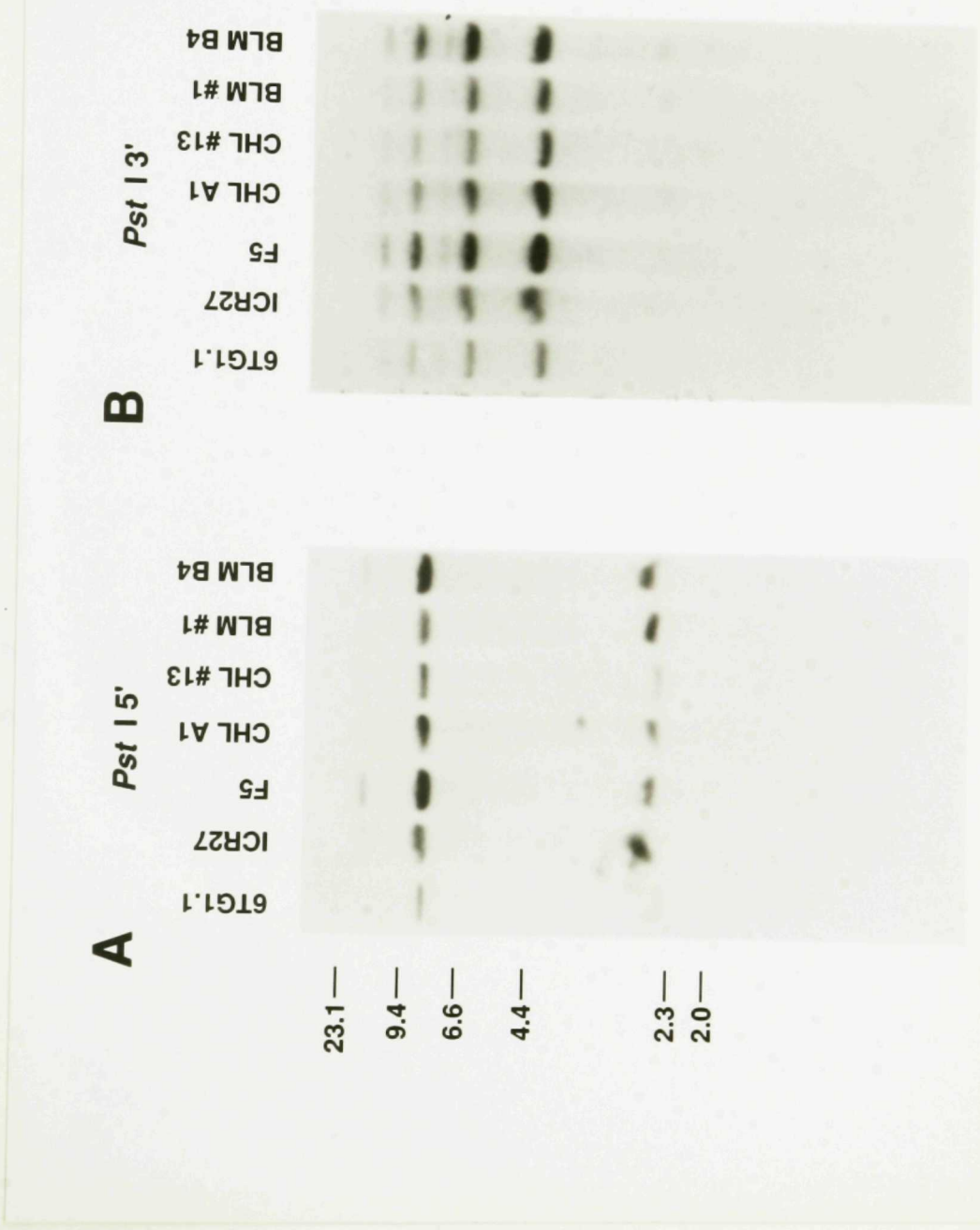

A
Pst I 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Pst I 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

Figure 35. Southern Analysis of Xba I digests.

Genomic DNA was digested to completion with Xba I, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.

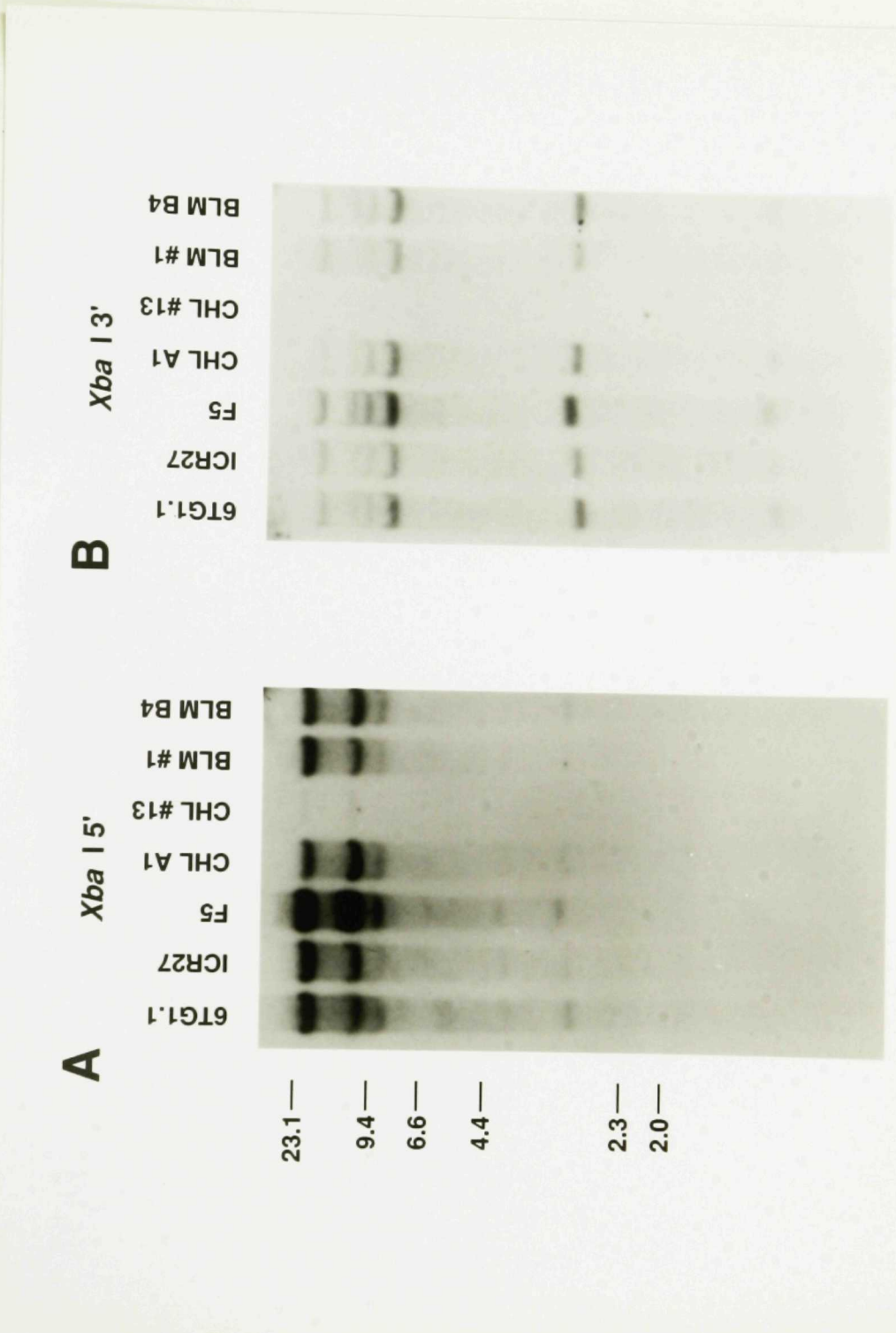
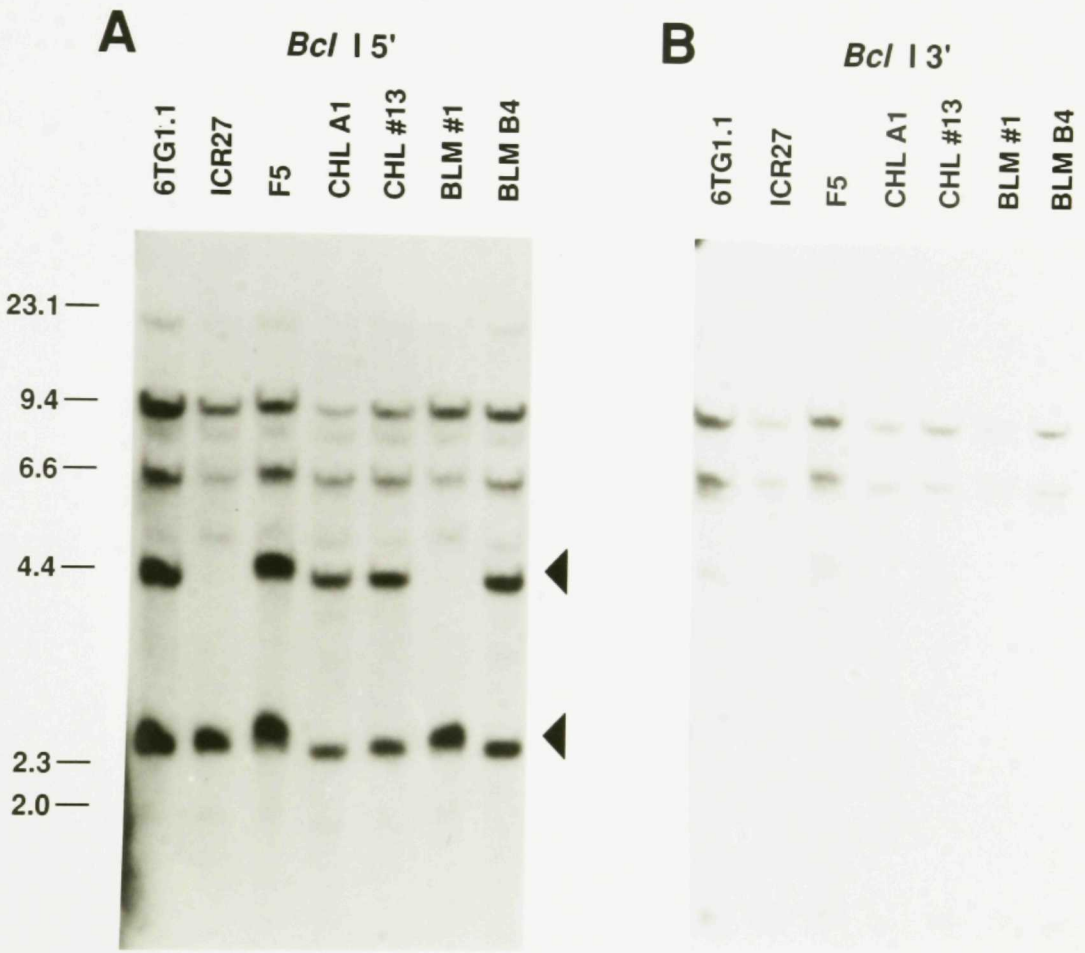

A
Xba I 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Xba I 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

Figure 36. Southern Analysis of Bcl I digests.

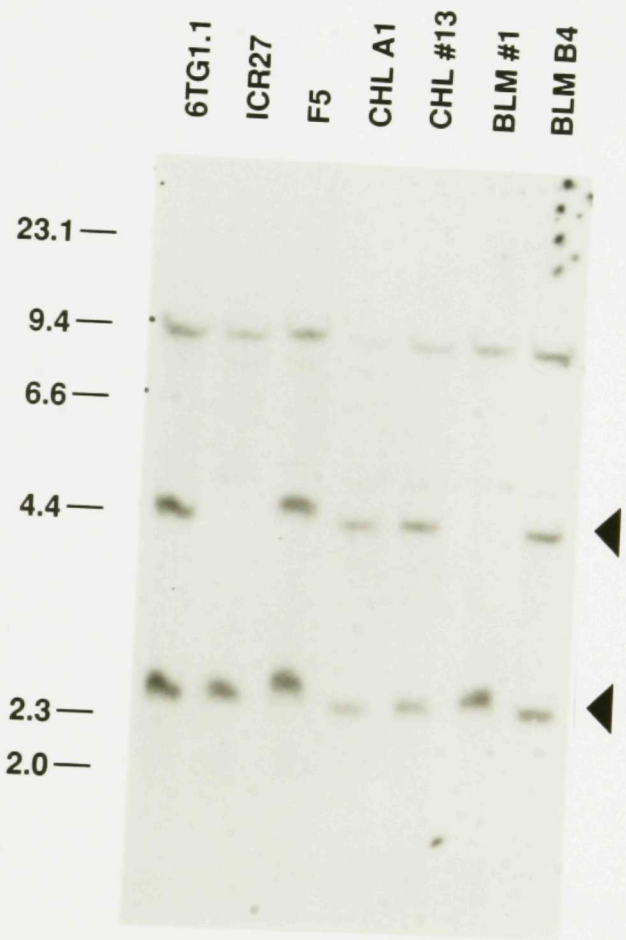
Genomic DNA was digested to completion with Bcl I, fractionated on a 0.8% agarose gel and transferred to Nytran filters as described in Methods. A.) Filters were probed with the 1.6 kb Pst I/Cla I fragment which contains sequence encoding part of the 5' untranslated region, amino terminal domain, first zinc finger, and the proximal portion of the second zinc finger. B.) Filters were stripped and reprobed with the 1.3 kb Cla I/Xba I fragment as described in Methods. The 1.3 kb Cla I/Xba I fragment contains the distal portion of the second zinc finger, the entire steroid binding domain and a portion of the 3' untranslated region.


A
Bcl I 5'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0
B
Bcl I 3'
6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4

digests of DNA isolated from dex^s cells contain both fragments, the results presented in Figure 36 suggest that parental dex^s cells are heterozygous for this polymorphism. In addition, they are consistent with the hypothesis that, at least in ICR27 and Blm #1 cells, drug-induced steroid resistance is the result of a deletion in the hGR gene encoding the GR^+ allele.

To attempt to map the site of this deletion, Bcl I digests were probed with a 402 bp Eco RI fragment of plasmid phGR2.9 (the same probe used to identify hGR mRNA) which contains sequences corresponding to exon 2 of the hGR (Zong et al., 1990). Results comparable to those obtained with the Pst I/Cla I probe were obtained (Figure 37); the 4.4 kbp Bcl I fragment was present in DNA isolated from dex^s parental cells, but not in samples isolated from the dex^r mutants ICR27 and Blm #1. These results therefore suggest that at least a portion of exon 2 has been deleted from the GR^+ allele in both of these mutants.

Figure 37. Mapping the site of the deletion present in ICR27 and Blm #1. The blot which was probed in Figure 36 was stripped and re-probed with the 402 bp Eco RI fragment of pHGR2.9 (Hollenberg et al., 1985). The 402 bp Eco RI fragment corresponds to residues 563-965 (C. Weinberger, personal correspondence) in Figure 30 and contains sequences specific for exon 2 of the human GR (Zong et al., 1990) Like the results seen in Figures 36, the 4.4 kb band was not detected in ICR27 and Blm #1.


6TG1.1
ICR27
F5
CHL A1
CHL #13
BLM #1
BLM B4
23.1
9.4
6.6
4.4
2.3
2.0

DISCUSSION

I. Identification and Characterization of Region-specific Anti-glucocorticoid Receptor Antibodies

Biochemical and molecular analyses of the GR have defined at least 3 functional domains: (1) an amino-terminal immunogenic domain, (2) a centrally located DNA-binding domain, and (3) a carboxyl terminal steroid-binding domain. The development of region-specific anti-GR antibodies could provide additional tools to examine the structure-function relationships of these domains. Therefore, we attempted to prepare region-specific anti-GR antibodies using synthetic peptide sequences corresponding to various regions of the rat GR. Of the 4 peptide sequences used, only the peptide corresponding to Cys_{500}-Lys_{517} generated an anti-GR antibody. This antibody, AP64, 1) recognized both the denatured and native forms of the GR in the rat and human, 2) preferentially interacted with the steroid-bound, activated, monomeric form of the GR, and 3) significantly blocked the binding of the activated GR to DNA.

Many anti-GR antibodies have been made against intact GR protein. Most of these antibodies interact with both unactivated and activated GR forms (Gametchu and Harrison, 1984; Eisen, 1980; Okret et al., 1981, Harmon et al., 1984). One characteristic of AP64 that distinguishes this antibody from those described above, is its ability to preferentially interact with the activated, monomeric form of the GR. A few of the anti-GR antibodies made against intact GR protein are able to discriminate between the various forms of the GR or affect GR function (Westphal et al., 1984; Robertson et al., 1987).

Like AP64, these antibodies must recognize specific structural and/or functional features of the receptor. However, unlike the situation for these antibodies, the epitope for AP64 is known, thus providing direct evidence that the region, Cys_{500}-Lys_{517}, is occluded in the non-steroid bound and steroid-bound unactivated forms of the GR.

In addition to the three major functional domains, the GR contains two nuclear localization signals, NL1, a 28 amino acid sequence closely associated with the DNA binding domain, and NL2, located within the steroid-binding domain (Picard and Yamamoto, 1987). The peptide used to make AP64 contains, in addition to the carboxyl terminal 6 residues of the core DNA binding domain, a portion of the putative nuclear localization signal NL1. Preliminary evidence suggests that the carboxyl terminal region of this peptide, which contains part of the nuclear localization signal, is essential for antibody recognition (Yen, P.M., and Simons, S. S., unpublished observations). Thus, it appears that at least a portion of the nuclear localization signal contained in the region Cys_{500}-Lys_{517} is physically occluded in the unactivated receptor.

Other antibodies, made against peptide sequences located in the distal zinc finger region of the DNA binding domains of the progesterone (Smith et al., 1988) and the glucocorticoid receptors (Smith et al., 1988; Wilson et al., 1988) have properties similar to those described for AP64. The 18 amino acid sequence used by Wilson et al. (1988) is amino terminal to the peptide used to make AP64. The 17 amino acid peptide of the progesterone receptor used by Smith et al. (1988) and the peptide used to make AP64 share only one common amino acid. In addition, the nuclear localization signal present in the GR, which

appears to be essential for AP64 recognition, is not conserved in the progesterone receptor. Since the epitopes of these three antibodies do not appear to overlap, it seems likely that these antibodies recognize different structural features of the receptor. Furthermore, the antibody BuGR-1 (Gametchu and Harrison, 1984) whose epitope is located amino terminal to the DNA binding domain (Eisen and Eisen, 1985; Rusconi and Yamamoto, 1987), recognizes both unactivated and activated GR forms. Taken together, these data provide evidence that there is a change in the availability of specific sequences involved in DNA binding activity and nuclear localization associated with GR activation. Thus, the process of activation may be multifunctional, exposing the DNA binding domain of the GR as well as regions involved in regulating its intracellular distribution.

AP64 defines a region of the GR that is occluded in the non-steroid-bound and steroid-bound unactivated forms. Yet upon GR activation, the topology of the GR changes, exposing this region of the receptor. Analysis of the structure-function relationships of the various domains in the GR indicate that the steroid binding domain may play a role in GR activation as well transcriptional activation (Webster et al. 1988). In the absence of ligand, this domain appears to be involved in the repression of transcriptional activity, since removal of the steroid binding domain results in a constitutively active receptor (Godowski et al., 1987).

There are two proposed models to explain how the steroid binding domain regulates transcription activity. Both of these models could also explain the occlusion of the epitope for AP64 in non-steroid-bound and steroid-bound unactivated GR. The "induction" model suggests that

the steroid binding domain covers regions necessary for DNA binding activity and transcriptional activation. Upon ligand binding, a conformational change occurs which unmasks regions necessary for DNA binding and transcriptional activation. Thus, the conformational change that occurs could also unmask the region of the GR recognized by AP64. However, it is difficult to reconcile this model with the fact that regulation of DNA binding activity and transcription activation by the steroid binding domain is independent of its location (Picard et al., 1988).

A second model suggests that the GR is associated with non-receptor components. In the absence of hormone, these receptor-associated proteins block regions of the GR that are necessary for DNA binding activity as well as transcriptional activation. Upon hormone binding, these receptor-associated proteins dissociate from the receptor exposing sites necessary for DNA binding activity and transcriptional activation. Thus, the association of these non-receptor components could also explain the occlusion of the epitope for AP64.

The most notable candidate for a receptor associated protein is hsp90. This protein is associated with the non-steroid bound and the steroid-bound unactivated GR (Sanchez et al., 1987a,b; Howard and Distelhorst, 1988) suggesting that the epitope for AP64 could be blocked by the presence of hsp90. Recent evidence shows that the site of hsp90 interaction with the rat GR is located in the steroid binding domain between residues 568 and 616 (Howard et al., 1990), downstream from the peptide sequence Cys_{500}-Lys_{517}. Thus, the presence of hsp90 may sterically hinder the interaction of AP64 with its epitope. On the other hand, hsp90 may be part of a protein complex that contains a 56-59

kDa protein (Sanchez et al., 1990; Renoir et al., 1990; Bresnick et al., 1990; Tai et al., 1986). Thus, it is possible that these proteins are responsible for the occlusion of the epitope for AP64. In any case, the data presented do not distinguish whether the epitope of AP64 is occluded due to a conformational change, or to dissociation of hsp90, hsp90-associated components, or non-receptor components yet to be discovered.

II. Biochemical characterization of the GR in $\underline{r}^+$ and $\underline{r}^-$ cells

The human GR gene is autosomal (Gehring et al., 1985; Hollenberg et al., 1985). We have previously proposed that in CEM-C7 cells, the GR genes are allelic (Harmon et al., 1989). The GR^+ allele encodes for a functional GR protein that can bind dexamethasone and dexamethasone mesylate, and is immunoreactive. GR^* encodes a non-functional GR protein which is immunoreactive and able to bind dexamethasone mesylate, but cannot, under physiological conditions, bind dexamethasone. Selection of dex^r cells after treatment with mutagenic agents results in cells expressing the $\underline{r}^-$ phenotype (Harmon and Thompson, 1981). Based on the characteristics of the GR present in dex^r $\underline{r}^-$ cells, it appears that mutagenesis results in a mutation(s) in the original functional allele (GR^+ GR^-), leaving the dex^r $\underline{r}^-$ cells with only the protein encoded by GR^*. In order to further define the distinguishing characteristics of the GR proteins encoded by GR^+ and GR^* alleles, the steroid binding properties of the GR present in dex^s $\underline{r}^+$ and the dex^r $\underline{r}^-$ cells were examined in cell extracts.

Initial analysis of the steroid binding properties of dex^s $\underline{r}^+$ 6TG1.1 and dex^r $\underline{r}^-$ ICR27 cells in cell extracts demonstrated the

following: 1) 6TG1.1 contained approximately twice as much steroid binding activity as ICR27 at 4°C in the absence of molybdate. In addition, there was no obvious difference in the equilibrium dissociation constants. 2) In contrast to the steroid binding activity seen in ICR27 at 4°C, steroid binding activity was essentially eliminated in ICR27 when assays were performed at 23°C, while steroid binding activity in 6TG1.1 was partially reduced. 3) The loss of steroid binding activity in ICR27 and 6TG1.1 at 23°C could be prevented by the addition of molybdate.

The results obtained in cell extracts showed significantly more binding activity in ICR27 cells than was predicted from the whole cell binding assays. However, it is difficult to compare the results obtained in intact cells with those obtained in cell extracts. The whole cell assay represents the physiological conditions present in the intact cell. In contrast, the broken cell assay may or may not be comparable to the conditions in the intact cell. In addition, in vitro assays measure specific processes, while assays in intact cells reflect the cumulative result of multiple processes (steroid binding, activation, nuclear translocation, etc.). Thus, the most valid comparison between intact and broken cell assays is probably obtained by comparing the results of the intact cell assays with those obtained in cell extracts at 23°C in the absence of molybdate, since under these conditions receptor activation is not inhibited. Based on the result of these experiments, it appears that the absence of binding seen in intact ICR27 cells might be the result of an activation-dependent process, rather than a defect in the steroid binding site of the protein encoded by the GR^* allele.

This hypothesis was supported by the ability of molybdate, a known inhibitor of GR activation (Leach et al., 1979; Dahmer et al., 1984), to enhance the binding in both ICR27 and 6TG1.1 extracts at 23°C. Unexpectedly, while molybdate had no apparent effect on ICR27 binding activity at 4°C, it appeared to have at least some effect on the binding to 6TG1.1 receptors at this temperature. It is possible that this effect is a property of the protein encoded by GR^+. Thus, similar effects would not be seen in ICR27. Alternatively, it is possible that this result is a function of experimental variation. In either case, the fact that molybdate stabilized the binding of ICR27 at 23°C is consistent with a role for activation in the instability of the protein encoded by the GR^* allele.

The hypothesis that loss of ligand from ICR27 receptors is activation-dependent was also supported by the results obtained when steroid dissociation from activated steroid-receptor complexes was examined. Dissociation kinetics showed that steroid was more rapidly lost from ICR27 than from 6TG1.1 receptors. The k_{off} for ICR27 was 0.056/min. Although the dissociation of steroid from 6TG1.1 receptors was curvilinear, both the fast (k_{off} = 0.032/min) and the slow (k_{off} = 0.0070/min) were slower than the k_{off} for ICR27. Since the proposed model suggests that the protein encoded by the GR^* allele is present in both r^+ and r^- cells, it was not obvious why the k_{off} of the fast component was not identical to the k_{off} of ICR27. One possible explanation for this apparent contradiction is that the receptors encoded by the GR^+ allele were only partially activated. In this case, the dissociation from the protein encoded by the GR^+ allele would itself be expected to be curvilinear since dissociation from activated GR has

been reported to be slower than dissociation from unactivated GR (Nemoto et al., 1989). Thus, the complex dissociation curve seen for 6TG1.1 GR may in fact reflect biphasic dissociation from the GR encoded by the GR^+ allele as well as the dissociation of the steroid from the GR^* allele.

In addition to the $\underline{r}^-$ phenotype, a second class of $\underline{dex}^r$ clones, $\underline{act}^1$, has also been isolated from CEM-C7 cells. $\underline{Act}^1$ mutants are characterized by their inability to retain bound ligand during activation of steroid-receptor complexes to the DNA binding form (Schmidt et al., 1980; Harmon et al., 1984). The ability of sodium molybdate to stabilize steroid-receptor interactions at 23°C, and the rapid loss of steroid under conditions favorable for GR activation, suggested that the GR protein present in ICR27 displayed many of the characteristics associated with the $\underline{act}^1$ phenotype. However, the fact that all $\underline{act}^1$ mutants have significant, albeit reduced, steroid binding activity when assayed in intact cells, while such activity is virtually absent in the $\underline{r}^-$ clone ICR27, suggests that the protein encoded by the GR^* allele differs to some extent from $\underline{act}^1$ GR. Indeed, in the presence of molybdate, dissociation of ligand from the GR present in the $\underline{act}^1$ mutant 3R7 was biphasic while dissociation from ICR27 GR was linear. Upon closer inspection, the k_{off} of the fast component of 3R7 (0.024/min) is similar to the k_{off} of ICR27 (0.037/min), implying that the protein encoded by GR^* while present in 3R7 can not be identical to the $\underline{act}^1$ receptor.

Taken together, these results suggest that the GR^* allele is present in $\underline{dex}^s$ CEM-C7 cells as well as in both $\underline{r}^-$ and $\underline{act}^1$ mutants. The dissociation experiments performed in 6TG1.1, ICR27, and IM-9 at 4 and 23°C in the presence of molybdate provided additional support for

this model. First, the dissociation curves for 6TG1.1 were biphasic, consistent with the idea that there are two different GR proteins being expressed in these cells, while the dissociation curves for ICR27 were linear. Second, the rates of steroid dissociation for the fast component in 6TG1.1 were similar to the rates of steroid dissociation for ICR27, implying that the GR present in ICR27 is also present in 6TG1.1. Finally, since the rates of steroid dissociation for the proteins encoded by the GR^+ allele (IM-9) and the GR^* allele (ICR27) could be readily determined, a theoretical dissociation curve for cells expressing both proteins could be constructed. This theoretical curve was found to be indistinguishable from the actual experimental data obtained from 6TG1.1. Therefore, it appears that 6TG1.1 is heterozygous, expressing proteins from two alleles, and ICR27 is functionally hemizygous, expressing only the protein encoded by GR^*. The rapid loss of steroid binding activity seen for ICR27 GR in the presence of molybdate was not due to receptor proteolysis, activation of the GR, or temperature-dependent denaturation of the steroid binding site. Thus, it appears that the protein encoded by the $\underline{r}^-$ allele contains an altered steroid binding site.

Despite the fact that rates of dissociation for 6TG1.1 and ICR27 were markedly different, Scatchard analysis failed to detect any significant differences in the K_ds for the receptors in the two cell lines. Nor did they detect the presence of two binding components in 6TG1.1. These results appear to be paradoxical since K_d is defined as the quotient of k_{off}/k_{on}. Since k_{off} is increased for the protein encoded by the GR^* allele, but the relative affinities for steroid are essentially identical in the two cell lines, it appears that there must

be a compensatory increase in k_{on} for the receptor encoded by the GR allele. Although it has been shown that the rates of steroid association for different steroid agonists are similar (Munck and Bell, 1974) increased rates of steroid association have been shown for the glucocorticoid antagonist dexamethasone oxetanone (Lamontagne et al., 1984). Therefore, it is possible that a mutation(s) in the GR could affect the structure of the steroid binding site in such a way as to alter both the rate of steroid association as well as steroid dissociation.

It is clear that the protein encoded by the GR^* allele expresses a complex phenotype. The fact that even in the presence of molybdate, dissociation of ligand is rapid suggests that it carries a mutation(s) in or near the steroid binding site. Although, the actual mutation(s) is unknown, it has recently been shown that the human estrogen cDNA clone, pOR8, obtained from MCF-7 cells encodes a mutant estrogen receptor whose affinity for estrogen is decreased at 25°C but not at 4°C (Tora et al., 1989). Inspection of the cDNA for pOR8 identified a point mutation in the hormone binding domain at residue 400 resulting in a glycine to valine substitution. Thus, it is possible that a comparable mutation exists in the GR^* allele and that the defect(s) seen in ICR27 is the result of a point mutation in the steroid binding domain. In addition, since binding activity is completely lost in extracts of ICR27 at 23°C in the absence of molybdate, and since the rate of dissociation of pre-bound ligand is even more rapid at 23°C in the absence of molybdate than in its presence, the protein encoded by the GR^* allele may also contain an $\underline{act}^1$ or $\underline{act}^1$-like mutation.

There is precedent for the presence of multiple mutations in mutant GR. Dissociation kinetics of the nt$^-$ clone S49.1TB.4.22R displayed both decreased DNA binding and nuclear translocation (Gehring and Tomkins, 1974), as well as a reduced affinity for steroid hormone (Spindler-Barth and Gehring 1982). Analysis of a cDNA clone from an nt$^-$ mutant showed the presence of two mutations. The mutation present in the DNA binding domain (arginine to histidine at residue 484) is responsible for decreased DNA binding and reduced nuclear translocation. The second mutation is located in the steroid binding domain (tyrosine to asparagine at residue 770). This latter mutation is responsible for the reduced affinity of the nt$^-$ mutant for steroid (Danielson et al., 1986). Although our results are consistent with the presence of more than one mutation in the GR* allele, formal proof will require the cloning and sequencing of this allele.

Support for the presence of two GR alleles in CEM-C7 cells has also been obtained using the non-conventional steroid cortivazol. Scatchard analysis of [^{3}H]cortivazol binding in cytosols prepared from dex^s r$^+$ (CEM-C7) cells revealed the presence of two binding components with high (0.4 nM) and low (11 nM) affinity (Schlechte et al., 1985). The receptor concentration of the low affinity site (0.30 pmol/mg protein) was approximately twice the amount of the high affinity (0.14 pmol/mg protein) site. Furthermore, similar analysis in cytosols prepared form dex^r r$^-$ (ICR27) cells showed only a single class of binding sites with affinity (0.8 nM) and concentration (0.13 pmol/mg protein) similar to the high affinity component seen in CEM-C7. In addition, cortivazol inhibited the growth of ICR27 cells and induced

glutamine synthetase activity, suggesting that the GR present in these cells could be activated by this novel pyrazolosteroid.

III. Dose Response and Mutation Induction of Adriamycin, Bleomycin and Chlorambucil

Glucocorticoids are often used in the treatment of lymphoproliferative diseases because of their known cytolytic effects on lymphoid cells (Claman, 1972; Goldin et al., 1977). Although single agent glucocorticoid therapy has been shown to be effective in inducing remissions (Viette et al., 1965; Wolff et al., 1967), resistance to single agent glucocorticoid therapy invariably occurs. Current therapies of lymphoproliferative diseases utilize multidrug treatment schedules which include the use of these steroids. Since many of the drugs used in these treatment schedules are potential mutagens, we examined the ability of adriamycin, bleomycin, and chlorambucil to induce steroid resistance in the steroid-sensitive human leukemic T cell line CEM-C7. Of the three drugs tested, only chlorambucil and high concentrations of bleomycin were found to induce steroid resistance in this model system.

Initial experiments examined the cytotoxicity of each drug on 6TG1.1 cells. The response to chlorambucil was log-linear, characteristic of simple alklyating agents. The response to adriamycin was more complex, with both a log-linear and plateau phase. The apparent insensitivity of a small fraction of the cells to high concentrations of adriamycin could be explained by the presence of an adriamycin-resistant subpopulation of cells. Alternatively, the plateau

could reflect the fact that during the 24 hour exposure there was a small fraction of cells which were not cycling. The response to bleomycin was clearly curvilinear. This appears to be typical of the response to this drug, as it has also been reported in mouse L5 cells (Terasima et al., 1972, 1979) and CHO cells (Barranco and Humphrey, 1971) The reason for this biphasic response in not clear. It is not the result of a mixed population of cells with differing sensitivities to bleomycin (Barranco and Humphrey, 1971; Drewinko et al., 1973; Terasima et al., 1979; Twentyman, 1984). Nor is it the result of metabolic breakdown of the drug (Terasima et al., 1979). Current theory suggests that cells become insensitive to the effects of the drug as a result of drug treatment, either through changes in cell permeability and/or drug transport, saturation of the target sites at the higher drug concentrations, or depletion of intracellular or extracellular cofactors required for drug action.

From the above dose response curves, concentrations were selected to determine if these drugs could induce mutations in the GR locus. Concentrations were chosen to attempt to kill 90% of the cells during a 24 hour exposure. The concentrations of adriamycin used to examine the acquisition of steroid resistance ranged from 0.25 μM-2.54μM. Peak plasma levels obtained in patients following intravenous infusion of adriamycin can reach concentrations of 0.1-1.0 μM (Ozols et al., 1982; Greene et al., 1983). Thus, the concentrations of adriamycin were comparable to clinically observed levels. Despite the fact that adriamycin has been shown to be a weak mutagen at the tk locus in L5178/$tk^{+/-}$-37.2 mouse lymphoma cells (Moore et al., 1987) and the hprt locus in V79 (Suter et al., 1980; Bhuyan et al., 1983; Wilson et al.,

1984) and CHO (Singh and Gupta, 1983) cells, it was not mutagenic at the GR locus in CEM-C7 cells.

Because of the biphasic nature of the dose response to bleomycin, two doses (2 μg/ml and 70 μg/ml) were chosen to examine the induction of steroid resistance. Depending on the route of administration, different plasma concentrations can be obtained. Bolus doses of 15 mg/m^2 result in peak plasma levels of 1-10 μg/ml (Calabresi and Parks, 1985), whereas continuous infusion of 40 mg/day can result in plasma levels reaching 150 μg/ml (Calabresi and Parks, 1985). Thus, depending on the route of bleomycin administration, both concentrations can be achieved clinically. Concentration dependent increases in chromosomal aberration have been reported following bleomycin treatment of HeLa and CCRF-CEM cells (Paika and Krishan, 1973) and of cultured lymphocytes (Ohama and Kadohtani, 1970; Dresp et al., 1978). We found that while low doses of bleomycin (2 μg/ml) did not induce mutations above background in the GR locus, high doses (70 μg/ml) were found to be weakly mutagenic. The mutagenic activity of high in contrast to the low doses of bleomycin could be attributed to the inefficient repair of damage resulting from the high doses.

The ability of high dose bleomycin to induce mutations leading to steroid resistance has also been examined in murine thymoma (S49) cells (Huet-Minkowski et al., 1981). In these cells, there was a dose dependent increase in the frequency of GR mutations with the maximum mutagenic effect occurring at 50 μg/ml. Furthermore, the dose of 70 μg/ml, which resulted in the induction of steroid resistance in CEM-C7 cells, was effective in killing all of the cells in the murine system.

Thus, it would appear that CEM-C7 cells are more resistant to the cytotoxic effects of this drug than S49 murine thymoma cells.

Chlorambucil is normally given orally, with the total daily dose being 4-10 mg (Calabresi and Parks, 1985). Peak plasma concentrations of this drug can reach approximately 1 μg/ml (Alberts et al., 1979; Mclean et al., 1979). Chlorambucil was found to be mutagenic at the GR locus in CEM-C7 cells at 5 μM (1.52 μg/ml), slightly higher than the clinically observed values.

IV. Characterization of the drug-induced steroid-resistant cells

The acquisition of steroid resistance has been studied in the steroid-sensitive human leukemic T cell line CEM-C7. To date, two major phenotypes have been characterized in this system: $\underline{r}^-$, which contains almost no steroid binding activity, and $\underline{act}^1$, which is unstable during the process of activation. Spontaneously arising steroid-resistant cells express the $\underline{act}^1$ phenotype, while the $\underline{r}^-$ phenotype had previously been seen in steroid-resistant cells isolated after treatment with the mutagenic agents ICR191 and MNNG (Harmon and Thompson, 1981). The present studies showed that both chlorambucil (5 μM) and bleomycin (70 μg/ml) induced mutations leading to steroid resistance in CEM-C7 cells. In both cases, the majority of the isolated steroid-resistant clones appeared to express the $\underline{r}^-$ phenotype.

However, not all of the steroid-resistant cells isolated expressed this $\underline{r}^-$ phenotype. In experiments performed with chlorambucil, 8 steroid-resistant clones were isolated. Of these, 2 did not express the $\underline{r}^-$ phenotype. In experiments examining steroid-resistant

clones after bleomycin treatment, 5 of 11 did not express the $\underline{r}^-$ phentotype. These results are not distinctly different from what would have been expected based on the mutagenic activity of the two drugs tested. Chlorambucil-induced steroid resistance 5-6 fold above background, while bleomycin-induced steroid resistance only 2.5 fold above background. Thus, one in five clones isolated after chlorambucil treatment and 2 in five clones isolated after bleomycin treatment would have been predicted to be the result of the background rate of spontaneous mutation (~ 10^{-5}/cell/generation; Harmon and Thompson, 1981). Since spontaneous mutation has been shown to yield mutants solely of the $\underline{act}^1$ variety (Harmon and Thomspon, 1989), all of which express significant levels of steroid binding activity, the results described here are entirely consistent with those previously reported.

As noted above, the ability of bleomycin to induce mutations leading to steroid resistance has also been examined in murine thymoma (S49) cells (Huet-Minkowski et al., 1981). Characterization of bleomycin-induced steroid-resistant cells isolated from murine cells showed that, unlike the $\underline{r}^-$ phenotype seen in human cells, a majority of the steroid-resistant cells contained greater than 25% of the steroid binding activity expressed in the parental cells. Many of these clones were shown to be $\underline{nt}^-$. Thus, while the precise nature of steroid resistance in human and murine cell lines differs, resistance is almost always due to a defect in GR function.

The biochemical and immunochemical characteristics of the GR in $\underline{r}^-$ drug-induced steroid-resistant cells were similar to those expressed in a previously isolated $\underline{r}^-$ cell line, ICR27. All cells had significantly more steroid binding activity when assayed in cell

extracts than in intact cells. The size of the GR protein as determined from affinity labeling and Western blotting was 92 kDa, indistinguishable from the GR in parental steroid-sensitive cells. In general, the 92 kDa protein appeared to be expressed in reduced amounts. In addition, the dissociation curves for two of the drug-induced steroid-resistant cells isolated, Chl#13 and Blm #1, were linear with k_{off}s equal to 0.049/min and 0.044/min, comparable to that seen in ICR27 cells (data not shown). Furthermore, the pattern of GR mRNA expression seen in the drug-induced steroid-resistant mutants, was comparable to that seen in ICR27 and other $\underline{r}^-$ mutants (Harmon et al., 1989). Thus, the $\underline{r}^-$ phenotype isolated after bleomycin or chlorambucil treatment displayed all of the characteristics of steroid-resistant cells isolated after mutagenesis with classical mutagens.

Based on our working model, it is assumed that in the selection of $\underline{r}^-$ cells, it is a mutation in the GR^+ allele which results in the loss of sensitivity to steroid. Examination of GR mRNA in $\underline{r}^-$ clones identified a 7 kb band in all cases. However, the amount of GR mRNA was somewhat variable, making it difficult to correlate mRNA levels with the reduced amounts of immunoreactive GR protein identified by Western blot analysis. The variability of GR mRNA levels is probably the result of clonal variation among the various $\underline{r}^-$ clones. Indeed such variability was seen in 5 $\underline{r}^-$ clones isolated after ICR191 mutagenesis (Harmon et al., 1989) In addition, Eisen et al. (1988) have shown that in 6TG1.1 cell GR mRNA is positively regulated by its cognate ligand. Thus, it is possible that small amounts of corticosteroid present in the culture medium could have influenced our results. However, cells used for the isolation of RNA were routinely grown in medium containing

charcoal striped fetal bovine serum. Since control experiments demonstrated that removal of steroid by this procedure was complete (data not shown) this possibility seems unlikely.

Based on our results it appeared that the $\underline{r}^-$ phenotype was the result of loss of mRNA expression from the GR^+ allele. To characterize the structure and organization of the GR genes in $\underline{r}^+$ and $\underline{r}^-$ cells, Southern blot analysis was performed on DNA isolated from $\underline{r}^+$ and $\underline{r}^-$ cells after digestion with a variety of restriction endonucleases. No differences were identified after digestion with Eco RI, Bam HI, Hind III, Pst I, and Xba I. However, in two of 5 $\underline{r}^-$ cell lines a 4.4 kbp Bcl I fragment was absent. Murray et al. (1987) had previously reported a Bcl I polymorphism in the human GR gene, resulting in fragments of 4.5 and/or 2.5 kbp. The presence of both the 4.4 kbp and the 2.5 kbp bands in steroid-sensitive $\underline{r}^+$ CEM cells suggests that these cells are heterozygous for this polymorphism. Based on preliminary results with an exon 2 specific probe, this deletion appears to contain a portion of exon 2, which encodes the amino terminal half of the GR (Zong et al., 1990). More importantly, the absence of this 4.4 kbp band in ICR27, as well as the bleomycin-induced steroid-resistant mutant Blm #1, suggests that there has been a deletion or rearrangement in the GR+ allele resulting in the $\underline{r}^-$ phenotype. Consequently it appears that the defective GR protein present in ICR27 cells must be encoded by GR^*. Our biochemical analysis would suggest that this protein contains a mutation(s) that affects the ability of the GR to retain steroid. Based on the domain structure of the GR, this mutation(s) would probably be located in the carboxyl terminal steroid binding domain.

As noted above, our preliminary results suggest that the deletion in the GR^+ allele results in the loss of exon 2. During the preparation of this thesis, the sequence of a portion of the GR gene was published (Zong et al., 1990). Analysis of this published sequence shows that the probe used to analyze GR mRNA was derived solely from exon 2 sequence. Thus, it is possible that the absence of smaller GR mRNA species on our Northern blots of RNA isolated from $\underline{r}^-$ cells was the consequence of the probe used rather than an actual lack of such species. However, when the blots shown in figure 29 were reprobed with the same 1.3 kbp Cla I/Xba I fragment used in figure 36, no shorter GR mRNA species were detected. Thus, it does not appear that such species are produced.

CCRF-CEM is a human lymphoblast cell line isolated from a female patient with acute lymphoblastic leukemia (Foley et al., 1965). Two of the clones isolated from CCRF-CEM are CEM-C1 and CEM-C7 (Norman and Thompson, 1977). Unlike CEM-C7 and its subclone 6TG1.1, CEM-C1 cells are resistant to the cytolytic effects of steroids (Norman and Thompson, 1977). Examination of the GR in CEM-C1 cells showed that unlike the biphasic dissociation curve seen in 6TG1.1, the dissociation curve for CEM-C1 GR was linear. This would imply that genes encoding the GR in CEM-C1 are homozygous or haploid. While previous reports suggest that the GR receptor in steroid-resistant CEM-C1 cells were normal (Zawydiwski et al., 1983), it should be noted that the rate of steroid dissociation for CEM-C1 (k_{off} -0.012/min) was not identical to either phase of 6TG1.1 (k_{off} slow = 0.0066/min, k_{off} fast = 0.0391/min). Thus, the GR present in CEM-C1 may not be identical to that encoded by the GR^+ allele present in 6TG1.1, suggesting that there might be more subtle defects in CEM-C1 receptors. Consequently, it is clear that CCRF-CEM is

actually a mixture of cells, arising either through drug treatment of the patient or growth in culture.

V. Concluding Remarks

A major goal of this work was to investigate the origins of glucocorticoid resistance in human leukemic cells and to evaluate the possibility that drugs commonly used in combination protocols for the treatment of leukemia and lymphoma might contribute to the acquisition of such resistance. Clearly, the results presented here demonstrate that glucocorticoid resistance is not a simple phenomenon attributable to a single mechanism. Instead, the phenotypes and genotypes of resistant cells are complex and multifaceted. Thus, while these studies provide new insight into the biochemical basis of various steroid resistant phenotypes, additional work will be required to determine, at the level of DNA sequence, the precise nature of the individual mutations responsible for steroid resistance.

Nevertheless, our studies clearly indicate that drugs commonly used in combination with glucocorticoids can induce mutations in the glucocorticoid receptor genes and that these mutations are probably similar to those induced by classical mutagens. Thus, the possibility must be considered that similar mutations may occur during patient therapy. In addition, our results suggest that not all CEM cells are created equal. It was previously shown that the uncloned cell line CCRF-CEM contains a heterogeneous population of sensitive and resistant cells (Norman and Thompson, 1977). Our results extend this observation to indicate that the allelic composition of the GR genes in these cells is also heterogeneous. Whether this heterogeneity is a result of long

term propagation of cells in culture, or represents heterogeneity of the original leukemia cannot be determined. However, the results presented here may ultimately provide the basis for direct examination of leukemic cells to ascertain the origins of diversity and resistance *in vivo*.

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